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Lecithin vesicular carriers for transdermal delivery of cyclosporin A

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Abstract

Two kinds of vesicles with and without the presence of sodium cholate (flexible vesicles and conventional vesicles) were prepared, using cyclosporin A as model drug. When applied onto the excised abdominal skin of mice non-occlusively, the enhancing effects of vesicles on the penetration of cyclosporin A were assessed by an in vitro permeation technique. The effect of sodium cholate micelles was also studied. In vivo study was carried out by topical application of vesicles onto the mice skin and drug serum concentration was detected. Results showed that after 8 h of administration, flexible vesicles transported 1.16 μg of cyclosporin A through per cm^2 mice skin and amounted to 1.88 μg 24 h later. The residual amount in the skin was 1.78 ± 0.51 μg/cm^2. However, flexible vesicles failed to transport measurable amount of drug through pre-hydrated skin while deposited 2.39 ± 0.26 μg/cm^2 into the skin. Conventional vesicles failed to transfer cyclosporin A into the receiver while accumulated 0.72 ± 0.19 μg/cm^2 of drug in the skin. Furthermore, 1 and 40% sodium cholate micelles precluded the transport of cyclosporin A. In vivo studies indicated that with the application of flexible vesicles, serum drug concentration of 53.43 ± 9.24 ng/ml was detected 2 h later. After the stratum corneum of mouse skin has been destroyed by shaving, flexible vesicles transfered large amount of drug into blood, up to 187.32 ± 53.21 ng/ml after 1 h of application. Conventional vesicles failed to deliver measurable amount of drug into the blood under normal skin condition. In conclusion, flexible vesicle is better than conventional vesicle as the carrier for transdermal delivery of cyclosporin A. Penetration and fusion have been suggested to be two major functional mechanisms. Hydration is detrimental to the enhancement effect. Stratum corneum constitutes main barrier to the transport of lipophilic cyclosporin A.

Keywords: Lecithin vesicles; Flexibility; Transdermal delivery; Cyclosporin A

1. Introduction

Nowadays, Various kinds of vesicular carriers have been suggested as vehicles for topical and transdermal delivery of drugs (Touitou et al., 1994; Cevc et al., 1996). Mezei and Gulasekharam

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were the first to report that liposomes loaded with triamcinolone acetonide facilitated a 3–5-fold accumulation of the drug within the epidermis and dermis (Mezei and Gulasekharam, 1980). Nonionic surfactant vesicles (niosomes) were introduced by Vanlerberghe et al. (1978) and were found to enhance the penetration of sodium pyrrolidone-carboxylate through stratum corneum (Vanlerberghe et al., 1978). Recently, two flexible lecithin vesicles-the ethosomes (Touitou et al., 1997) and the transfersomes (Cevc et al., 1997) have been reported to enhance transdermal delivery of water-soluble drugs when applied onto the skin non-occlusively.

Although the use of vesicles in dermal or transdermal drug delivery has been extensively studied, little is known about the influence of vesicle-skin interaction on the permeation of lipophilic drug with large molecular weight through stratum corneum both in vitro and in vivo.

We have investigated the effect of detergent on the flexibility of lecithin vesicles (Guo et al., 1999). Flexibility was determined by permeation through membrane barrier under an external pressure. The relative permeation efficiency was compared with water. Results showed when the cholate to lecithin weight ratios rised from 0 to 0.28, the relative permeation efficiency increased from 0 to 85%. The vesicles without cholate were rigid while vesicles containing cholate were much more flexible. In this work, we intended to study the effect of conventional and flexible lecithin vesicles on the in vitro and in vivo transdermal delivery of lipophilic polypeptides, and to shed some light on the mechanisms of vesicular functions. The effect of sodium cholate micelles on the permeation of drug was also studied for comparison. A nonpolar cyclic oligopeptide consisting of 11 amino acids, cyclosporin A was chosen as model drug.

2. Materials and methods

2.1. Chemicals

Soybean lipid (purity $>80\%$, lot number: 980607) was purchased from Shanghai Fuda pharmaceutical manufacturing factory (China) and lecithin was freshly prepared by column chromatography on aluminium oxide according to the reported method (Singelton et al., 1964). Only lecithin with purity $>95\%$ was used in this study. Sodium cholate was purchased from Serva (purity $>97\%$, lot number: 960426). Cyclosporin A powder (USP 23, lot number: 040396) was obtained from Galena (CZECH Republic). Methanol, chloroform, sodium chloride and other reagents, all p.a., were products of Nanjing Chemical (China).

2.2. Preparation of vesicles and micelles

Two kinds of vesicles were prepared by a conventional rotary evaporation-sonication method. Appropriate amounts of lecithin (7.5 wt.%) and cyclosporin A (3.75 wt.%) were dissolved in cosolvent of methanol and chloroform (1:1). When sodium cholate was used, the weight ratio of surfactant and lecithin was 0.28 so that the total weight of compositions remained equal. The mixture was dried to a thin film under vacuum. The film was then hydrated with 0.9% NaCl solution to make a lipid coarse suspension. Under room temperature (25°C), sonication was carried out (JY 92-II ultrasonic processor, China) to obtain small vesicles ($r<100$ nm).

Sodium cholate (1%), equivalent of formulation amount, was added to the 0.9% NaCl solution containing 3.75% of cyclosporin A. The mixture was vigorously shaken, put in ultrasonic bath for 15 min and stayed for 72 h at 37°C until equilibrium was reached. After filtration to discard undissolved solute, the concentration of cyclosporin A was determined by HPLC. A total of 40% of sodium cholate was required to dissolve 3.75% of cyclosporin A completely, which resulted in rather viscous solution. Both micelles were assessed for the permeation of cyclosporin A.

2.3. Characterization of vesicles

Diameter and polydispersity(V) of vesicles were measured by a Zetamaster instrument (Malvern Instruments, Malvern, UK).

Samples for transmission electron microscopy (TEM) were prepared at room temperature by conventional negative staining methods using 1% phosphotungstic acid buffer (pH 6.0). Samples were viewed on an H-7000 model transmission electron microscope (Hitachi, Japan).

2.4. HPLC assay

Concentration of cyclosporin A was determined by HPLC. The HPLC system was consisted of a pump (Model LC-5A, Shimadzu, Japan), a shimpack CLC-ODS column (150×6 mm ID, Shimadzu) maintained at 70°C, an UV detector (Model SPD-6A, Shimadzu, Japan) at 210 nm and a data station (Model C-R6A, Shimadzu, Japan). The mobile phase was composed of 72% (v/v) methanol and 28% water, and delivered at a flow rate of 1.2 ml/min. The injection volume was 20 μl and the relative retention time was found to be 11.0 min.

2.5. In vitro transdermal delivery studies

2.5.1. Preparation of skin samples

Kunming mice, 3–4 weeks old, weighed 18–22 g, were sacrificed. The hair of test mice was carefully trimmed short (< 2 mm) with a pair of scissors and the abdominal skin was removed from the mouse body. The skin was used directly without further treatment. To investigate the influence of hydration, some of the skin samples were pre-hydrated overnight (12 h) in 0.9% sodium chloride solution.

2.5.2. Permeation experiments

A modified Franz diffusion cell with a diffusion area of 2.01 cm^2 was used. We have compared the effect of isotonic saline, 20 and 50% ethanol as diffusion medium. When saline alone was used, cyclosporin A was barely detectable because it has poor solubility in water. The equilibrium solubilities of cyclosporin A in 0.9 saline, 20 and 50% ethanol were determined to be 0.18 ± 0.02 μg/ml, 60.61 ± 3.96 and 167.24 ± 7.37 μg/ml ($n = 3$), respectively. Ethanol (20%) was demonstrated to be the most appropriate considering the sink condition and skin physiological activity. Therefore, the receptor cell was filled with 16.5 ml 20% ethanol adjusted to isotonic by 0.9% NaCl, followed by mounting and fixing the mouse skin on the receptor cell. A total of 20 μl of conventional vesicles (with cyclosporin A content of 3.33 mg/ml) and flexible vesicles (with cyclosporin A content of 3.45 mg/ml) were respectively applied onto the 2.01 cm^2 of skin and left to dry out. A total of 1 and 40% cholate micelles (with cyclosporin A content of 0.08 and 3.30 mg/ml, respectively) were similarly administered. For comparison, flexible vesicles were also applied onto the pre-hydrated skin. The experiment was performed at 37 ± 0.5°C for 24 h with constant magnetic stirring. A sample of 2 ml at each designated time interval was withdrawn for HPLC analysis. The removed sample was immediately replaced with the same amount of diffusion medium and corrected according to the dilution proportion.

2.5.3. Extraction of cyclosporin A in skin

At the end of the permeation experiments, the surface of the skin was washed thrice with 50% ethanol, then with water to remove excess drug on the surface, and cut into small pieces. The tissue was further homogenized with 2 ml of water. The homogenates were transferred to a 10-ml conical tube and extracted with 2.5 ml of ether and 2.5 ml of hexane. After shaking for 5 min and centrifuging for 5 min at 2000 rpm, the organic phase was transferred to a 5-ml conical tube for evaporation to dryness under a stream of nitrogen at 35°C. Residue was redissolved in 100 μl of the mobile phase and 150 μl of hexane, shaken for 1 min and centrifuged for 5 min at 2000 rpm. An aliquot of 20 μl was injected into the chromatography.

To validate skin washing procedures, as control, 20 μl of vesicles containing cyclosporin A were administered onto the skin surface and washed immediately after drying out. The drug content in the skin was subsequently determined.

2.6. In vivo animal tests

Kunming mice, 3–4 weeks old, weighed 18–22 g, were kept under normal laboratory conditions. The hair of test mice was carefully trimmed short with a pair of scissors. To destroy the integrity of

stratum corneum, the other group of mice was shaved with scraper. The application area on the abdominal skin was limited to 2 cm^2. Flexible vesicles and conventional vesicles (10–30 μl, according to the body weight of mice) were respectively administered and left to dry out.

Mice were respectively sacrificed after 1, 2, 4, 6, 8, and 12 h of application. Serum concentration followed the same procedure of extraction was detected by HPLC.

3. Results

3.1. Physical properties of vesicles

All of the lipid coarse suspensions obtained were turbid. Both conventional and flexible vesicles were transparent colloidal dispersions, average diameter being 74.0 and 61.8 nm, respectively. The polydispersity index of the former was 14.6%, indicating the formation of almost monodisperse particles, while that of the latter being 50.6%.

Fig. 1 showed the transmission electron photomicrographs of both kinds of small vesicles, respectively. It was evident that the particles shown in these photographs were approximately spherical liposomes with obvious whorls like fingerprints. There was no significant difference of appearance between these two small vesicles. Studying the TEM validated the results of particle sizes measurements.

3.2. Determination of cyclosporin A

The calibration curve for cyclosporin A content in receptor solution exhibited good linearity from 0.1 to 4 μg/ml ($r^2 = 0.9999$). The regression equation was $C = 1.33 \times 10^{-4}A + 9.93 \times 10^{-3}$, where C (μg/ml) and A represented the concentration and peak area of cyclosporin A, respectively. The recovery data was $97.4 \pm 5.9\%$ ($n = 3$).

The calibration curve for cyclosporin A content in the skin also exhibited good linearity from 0.01 to 3.93 μg/cm^2 ($r^2 = 0.9998$). Limit of determination was 10 ng. The regression equation was $C = 5.97 \times 10^{-6}A + 3.59 \times 10^{-3}$, where C (μg/cm^2) represented the content of cyclosporin A per unit area of skin. The recovery data was $94.4 \pm 6.2\%$ ($n = 3$).

The standard curve was produced for serum concentration ranging from 50 to 625 ng/ml ($r^2 = 0.9991$). The regression equation was $C = 3.91 \times 10^{-2}A - 1.17$, where C (ng/ml) represented the serum concentration of cyclosporin A. The recovery data was $96.1 \pm 5.7\%$ ($n = 3$).

Precision assay showed all the relative standard deviations within 1 day and among every other day were less than 10%.

3.3. In vitro and in vivo permeation

As seen from Fig. 2, flexible vesicles delivered 1.16 ± 0.26 μg of drug through per cm^2 of mice skin into the receiver after 8 h of permeation and

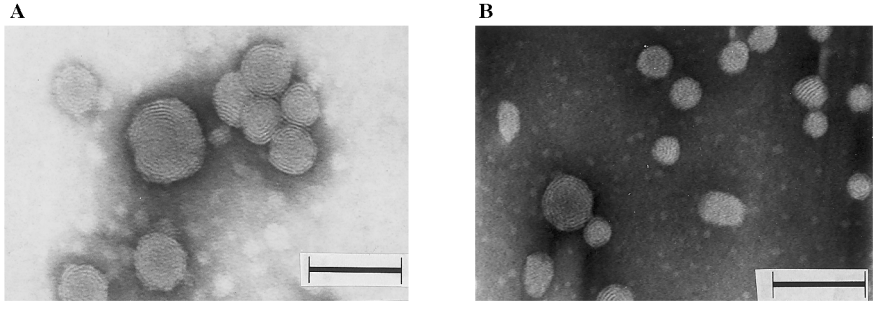

Fig. 1. Transmission electron photomicrographs of vesicles with (A) and without (B) the presence of sodium cholate (× 180 000). Bar is 100 nm.

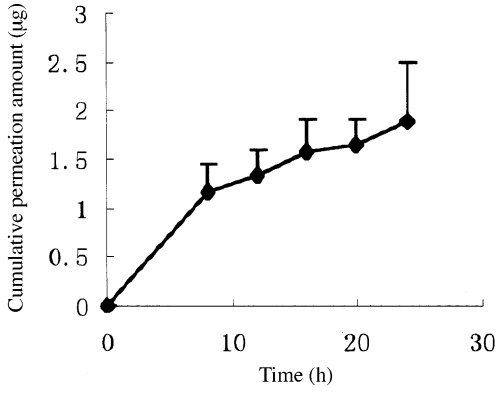

Fig. 2. Cumulative amounts of cyclosporin A permeation from vesicles with the presence of sodium cholate through per cm^2 of the mice skin in vitro. Data are means $\pm$ S.D., $n = 6$.

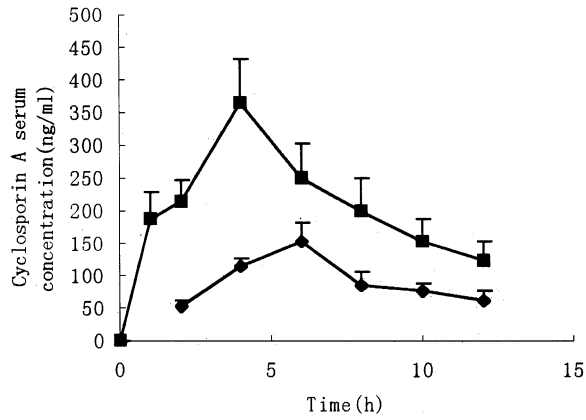

Fig. 3. Cyclosporin A serum concentration as function of time in murine blood after an epicutaneous administration of flexible vesicles under normal (◆) skin and shaved (□) skin condition.

amounted to 1.88 ± 0.06 μg 24 h later. There must be certain amount of drug transported before 8 h, but the concentration was below the limit of determination. On the other hand, conventional vesicles and micelles failed to transfer cyclosporin A across the mice skin after 24 h of permeation.

Table 1 showed the residual amount of cyclosporin A in the skin after administration of different preparations. Compared with control blank sample and both micelles, both flexible and conventional vesicles transported fairly large amount of cyclosporin A into the mice skin, totaling 1.78 ± 0.51 and 0.72 ± 0.19 μg/cm^2, respectively. The difference between the former three samples and vesicles was extremely significant ($P < 0.01$). Furthermore, there existed extremely significant difference between both vesicles ($P < 0.01$).

Flexible vesicles failed to transport measurable amount of cyclosporin A through hydrated skin while deposited 2.39 ± 0.26 μg/cm^2 of cyclosporin A into the skin.

In vivo study showed that with the application of flexible vesicles, serum drug concentration of $53.43 \pm 9.$ 24 ng/ml was detected after 2 h of permeation and amounted to 154.37 ± 27.15 ng/ml 6 h later (Fig. 3). When the stratum corneum of mouse skin has been destroyed by shaving, flexible vesicles transferred large amount of drug into blood, up to 187.32 ± 53.21 ng/ml after 1 h of application and amounted to 364.51 ± 54.06 ng/ml 4 h later. Conventional vesicles failed to deliver measurable amount of drug into the blood under normal skin condition.

4. Discussion

Transdermal delivery of cyclosporin A is never a simple task as a result of its highly lipophilic nature, large molecular weight and a ring structure. Even dermal delivery of cyclosporin A to

Table 1
The residual amount of cyclosporin A in the skin after administration of different preparations[a]

Preparations	Control	1% cholate micelles	40% cholate micelles	Vesicles without cholate	Vesicles containing cholate
The amount of Cyclosporin A in the skin (μg/cm^2)	0.11 ± 0.05	0.06 ± 0.02	0.15 ± 0.08	0.72 ± 0.19	1.78 ± 0.51

[a] Data are means $\pm$ S.D., $n = 6$.

treat psoriasis has not obtained successful result yet (Wang et al., 1997a). Wang et al. (1997b) has applied electroporation technique, a single-pulse mode at 200 V/cm, $\tau = 10$ ms resulted in delivering 87 ng of cyclosporin A in 0.87cm^2 of rat dermis and less than 0.010 μg in 0.8 ml of receiver. Other techniques have also been tried, including iontophoresis and pretreatment with penetration enhancers, etc. (Choi et al., 1995). When iontophoresis was applied, with increasing constant voltage from 1 to 5 V, the permeation of cyclosporin A through per cm^2 mice skin increased from 0.375 to 2.50 μg (Wang et al., 1997a). Most of enhancers have little effect despite 5% DMSO and 1% Azone (Choi et al., 1995). But the utilization of enhancers usually involves the pretreatment of skin, which might inevitably change the nature of skin.

From our results, it was evident that lecithin vesicles with the incorporation of sodium cholate promoted the transfer of cyclosporin A through the mice skin while sodium cholate micelles and the vesicles without cholate failed to achieve. The particle size distribution of both vesicles was similar while polydispersity was different. The smaller polydispersity of flexible vesicles indicated the formation of monodisperse particles, probably because the addition of sodium cholate increased the fragility of vesicles and made the particle size distribution more uniform.

Compared with micelles, both vesicles resulted in fairly large amount of drug deposited in the skin. It appears lipid vesicles are capable of adsorption and fusion in the stratum corneum (Abraham and Downing, 1990; Callaghan et al., 1990), which might promote the accumulation of drugs.

Sodium cholate have long been used to extract cell membrane proteins (Almog and Lichtenberg, 1988), so it is possible to function as permeation enhancer. But from our results, sodium cholate itself cannot enhance the permeation of cyclosporin A at either 1 or 40% concentration. Therefore, the enhancement effect was not as a result of the extraction of membrane protein and the micelles probably precluded the transfer of drug to the skin surface. The effect of vesicles without cholate was inferior to that of vesicles containing cholate. It is natural to conclude the presence of sodium cholate affects the vesicular membrane properties. The synergetic effect of lecithin and cholate facilitates the penetration of the vesicles into the skin and there exists two main reasons for the function of flexible vesicles. On one hand, the flexible vesicles containing sodium cholate were capable of penetrating through the interstices of stratum corneum under the influence of transcutaneous hydration force caused by the water concentration difference between the skin surface and skin interior (Cevc and Blume, 1992). The hydration of skin decreased the water concentration difference thus precluded the transport of drug through skin. While hydration loosened the interstices, which was beneficial for the accumulation of lipophilic cyclosporin A. On the other hand, fusion of vesicles with skin also contributed to the enhancement effect (Kirjavainen et al., 1996). Sodium cholate has been demonstrated to influence the physicochemical properties of phospholipid bilayers, exerting a strong perturbation on the lecithin alkyl chain order and giving a large decrease of the order parameter (Ulmius et al., 1982). The increase in the fluidity of bilayers might lead to the increase of fusion. Furthermore, it has been reported membrane ripples were found after the addition of cholate in the lecithin vesicles, whose surfaces were smooth from freeze fracture electron microscopy. These ripples may serve as intermembrane attachment sites for membrane fusion (Schubert et al., 1986). Therefore, the fusion effect of vesicles containing cholate with skin lipid was greater than that of vesicles without cholate.

In vivo study showed flexible vesicles transproted measurable amount of cyclosporin A into the blood circulation after 2 h of application while conventional vesicles failed. When the stratum corneum of mouse skin has been destroyed, flexible vesicles transfered large amount of drug into blood after 1 h of application, which demonstrated stratum corneum constituted main barrier to the transport of lipophilic cyclosporin A by flexible vesicles.

Although the precise mechanisms by which vesicles containing sodium cholate exert their functions have yet to be thoroughly explored, the

current results promise well in the transdermal delivery of drugs.

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