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Topical delivery of different acyclovir palmitate liposome formulations through rat skin *in vitro*

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The objective of this investigation was to examine the permeation of acyclovir palmitate from various liposome formulations through hairless rat skin *in vitro*. The penetrated amount, permeability and intradermal retention of ACV-C_{16} were compared among various lipid compositions and different vesicle charges. We found that all of the liposome formulations resulted in higher flux and permeability of ACV-C_{16} than a common ointment form. The 'skin lipid' liposome provided the most effective transdermal delivery of incorporated ACV-C_{16}. Presence or absence of cholesterol in the lipid bilayers did not reveal any difference in transdermal delivery of the associated ACV-C_{16}. Intradermal retention of ACV-C_{16} from positive liposomes was significantly higher than that from other formulations. These findings suggested that liposomes itself might not penetrate through the skin, but enhance the transfer of incorporated ACV-C_{16}. Liposomal lipid composition was the most important factor affecting the efficiency of transdermal delivery of incorporated drugs, but was not correlated with its phase transition temperature.

1. Introduction

In the formulation of topical dosage forms, great attention has been devoted to new structures which can both ensure adequate intradermal retention of the drug within the skin to enhance the local effect and reduce the percutaneous absorption of the drug through the stratum corneum and viable epidermis into the central blood supply for a systemic effect (Schreier and Bouwstra 1994). For these purposes vesicular systems, liposomes in particular, have been investigated by several groups (Vutla et al. 1996; Egbaria and Weiner 1990). In a number of instances, liposomal drug formulations have been shown to be markedly superior to conventional dosage forms, especially for topical drug administration. The major advantages of topical liposomal drug formulations are their ability: (1) to reduce serious side effects and incompatibilities that may arise from undesirably high systemic absorption of drug; (2) to enhance significantly the accumulation of drug at the site of administration as a result of the high substantivity of liposomes with biological membranes; and (3) to readily incorporate a wide variety of hydrophilic and hydrophobic drugs. Liposomes are also non-toxic, biodegradable and are readily prepared on a large scale.

As a topical antiviral drug, acyclovir (ACV) has especially high potential for the treatment of herpes, condylomata acuminata, and other similarly manifested viral disease states. When systemic routes of administration are used to deliver ACV to the living epidermal tissues of the skin and membrane in which virus replication and lateral spread occur, sufficient ACV must be administered to account for the distribution of the drug through all tissues of the body. Therefore, systemic regimens adequate to suppress skin symptoms often result in systemic adverse effects and still may not overcome the inaccessibility of the target tissue to the drug. The use of liposomes as a topical ACV delivery system provides a perfect solution to the effective treatment of viral infection diseases.

To overcome the shortcomings of low encapsulation efficiency and rapid leaking of ACV liposomes, the prodrug ACV palmitate (ACV-C_{16}) was synthesized by esterifying the 2-hydroxy group of ACV as palmitate. As a result of structural modification, the n-octanol/water partition coefficient (logP) and ionization constant (pK_a) were increased significantly from – 0.4 to 8.7 and from 5.42 to 7.26, respectively. In this study, ACV-C_{16} was selected as a model drug. Aim of the investigation was to evaluate, by means of *in vitro* permeation experiments through hairless rat abdominal skin, the percutaneous behavior of liposome preparations that were affected by the changes in lipid compositions and the surface charge of liposomes in comparison with common ACV-C_{16} ointment and to investigate the mechanism by which liposomes facilitated the deposition of drugs into the skin.

2. Investigations, results and discussion

2.1. Physical characteristics of different liposome formulations

Zeta potential, mean diameter, polydispersity and pH of the liposomes were the parameters chosen to indicate the physical stability of liposomes. Measurements were performed immediately after preparation of liposomes. The results are shown in Table 1.

Table 1: Physical characteristics of liposomes of different lipid compositions

Type	Lipid compositions and ratio	Zeta potential (mV ± SD)	Mean diameter (μm ± SD)	Polydispersity ($\bar{x}$ ± SD)	pH
Skin (−) liposome	CA/CH/CS/PA (4/2.5/2.5/1) (w/w)	85 ± 7.2	0.62 ± 0.06	0.18 ± 0.01	6.74
Negative (−) liposome	PC/CH/PS (1/0.5/0.1) (M/M)	82 ± 6.4	0.64 ± 0.04	0.16 ± 0.01	7.33
Positive (+) liposome	PC/CH/SA (1/0.5/0.1) (M/M)	90 ± 4.8	0.54 ± 0.05	0.23 ± 0.02	7.88
Neutral (○) liposome	PC/CH (1/0.5) (M/M)	58 ± 2.3	0.51 ± 0.03	0.20 ± 0.01	7.23
DPPC (○) liposome	DPPC/CH (1/0.5) (M/M)	50 ± 1.9	0.50 ± 0.03	0.25 ± 0.02	7.37
PC (○) liposome	PC	49 ± 1.1	0.57 ± 0.04	0.20 ± 0.01	7.15

Some studies reported that a zeta potential of more than 60 mV usually indicated excellent stability, 40–60 mV good stability, 30–40 mV moderate stability and 10–30 mV incipient instability (Schreier et al. 1997; Zimmermann and Müller 2001). The results of our study showed that CA/CH/CS/PA, PC/CH/PS and PC/CH/SA were the most stable of the different liposome preparations. No significant differences in mean diameter were observed among various lipid compositions and different vesicle charges. The pH of the skin lipid liposome was the lowest of all the systems tested. This could be expected, since it contained PA that would lower the pH of the system. In our pilot study, although some change in pH occurred, no pH lower than 6.4 was found, not even after storage for 6 months at 25 °C (data not shown).

2.2. Effects of vesicle charge on percutaneous penetration of ACV-C_{16}

The skin permeation and retention of ACV-C_{16} from various liposome formulations are measured and summarized in Table 2 and Fig. 1.

Table 2 shows that the penetrated amount and flux of ACV-C_{16} were not statistically different comparing different surface charges among various liposomal formulations except for skin liposomes (CA/CH/CS/PA), but the skin retention of ACV-C_{16} was different. The positive liposomes showed significantly higher ($P < 0.05$), and the neutral liposomes showed a significantly lower, skin retention of the active ingredient than others of the liposomes. The results supported the hypothesis (Egbaria et al. 1990) that positive liposomes had an advantage over neutral or negative liposomes because at physiological pH the cell surface bore a net negative charge. Despite these advantages, we do not recommend positive liposomes as optimum liposomal formulations in enhancing percutaneous penetration of entrapped drug because they were more irritating and more unstable than neutral or negative liposomes.

Table 2: Permeation and retention of ACV-C_{16} from various liposome formulations through rat skin after 48 h

Sample	[a] Q_{cum} (% of dose)	Flux μg cm^{-2} h^{-1}	Retention (% of dose)
CA/CH/CS/PA	[b] 4.93±0.21	[c] 4.47±0.18	[d] 1.67±0.05
PC/CH/PS	2.54±0.15	1.24±0.05	0.89±0.04
PC/CH/SA	3.11±0.14	1.26±0.02	[d] 1.82±0.09
PC/CH	2.97±0.10	1.04±0.04	0.93±0.02
DPPC/CH	2.21±0.07	0.75±0.03	0.78±0.02
PC	1.82±0.05	0.51±0.02	0.33±0.02
Ointment	1.67±0.03	0.37±0.03	0.20±0.01

CA, bovine brain ceramide; CH, cholesterol; CS, cholesteryl sulfate; PC, egg lecithin; PA, palmitic acid; SA, stearylamine; PS, phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine

[a] Q_{cum}: cumulative amounts of permeated formula (±SD) after 48 h

[b, c] Significant difference ($P < 0.01$), comparing among all the others

[d] Significant difference ($P < 0.05$), comparing with neutral or negative liposomes

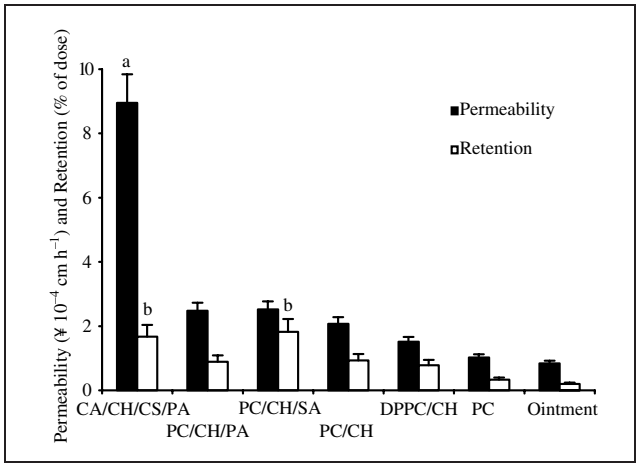

Fig. 1: Permeability and retention of ACV-C_{16} from various liposome formulations, [a] Significantly different ($P < 0.01$), comparing among all the others, [b] Significantly different ($P < 0.05$), comparing among all the others except one another

Fig. 1 shows that that liposomal ACV-C_{16} formulations were superior to conventional ointment formulations in facilitating drug retention in, and permeation through rat skin. Lipid compositions of liposomes significantly influenced the transdermal activities of entrapped ACV-C_{16}. Liposome formulations devoid of cholesterol did not show significant alterations in skin permeation and retention of ACV-C_{16} compared with ointment. Liposomes consisting of skin lipid composition more significantly ($P < 0.01$) enhanced the skin permeation of ACV-C_{16} than other liposome formulations. Skin retention of ACV-C_{16} from skin lipid liposomes was comparable with that from positive liposomes, and was significantly higher ($P < 0.05$) than from other liposome formulations.

Unlike all other biologic membranes, the stratum corneum has shown to be devoid of phospholipids. It consists primarily of CA (40%), CH (25%), fatty acids (primarily PA, 25%) and CS (10%) (Bouwstra et al. 1991). Lipid mixtures similar to those given above have been proved to form bilayer vesicles at physiological pH (Wertz et al.

1986). These skin lipid liposome data were particularly encouraging, since no attempt was made to optimize the lipid ratios. The best result exerted by the skin lipid liposome might come from its optimum miscibility with the lipid bilayers of the skin, and thus might facilitate the release of ACV-C_{16} from formulation and transport of ACV-C_{16} through skin.

No liposomal lipid was found in the receptor compartment (data not shown). The result indicated that a whole liposome vesicle did not penetrate intact through the skin at all, but merely facilitated transfer of ACV-C_{16} having been incorporated in liposomal bilayers through the skin. Phospholipids possess the property of surfactants which were thought to exert a "pull" effect on the membrane, i.e. to penetrate into the intercellular lipid bilayers (where the barrier function of the skin was located), thereby reducing the crystallinity of the intercellular lipid bilayers and thus increasing the permeability of these bilayers.

2.4. Effects of cholesterol on percutaneous penetration of ACV-C_{16}

Cholesterol can alter the fluidity of the liposomal membrane both above and below the phase transition temperature (T_c): it decreases the fluidity above this temperature, and increase the fluidity below this temperature. ACV-C_{16} liposomes consisting of PC with or without cholesterol were compared in order to investigate whether the fluidity of liposomal membranes was responsible for the differences observed from different lipid compositions. The results (Table 2) show that fluidity of the lipid compositions might be not an important factor for determining permeation and retention of the entrapped ACV-C_{16}, and the higher ACV-C_{16} permeation from PC ($T_c = -7\,^\circ C$) than DPPC ($T_c = -41\,^\circ C$) with cholesterol might not be due to better fluidity of PC. This finding was further supported by the result observed that skin-lipids, with higher T_c (87.9 °C) than PC and DPPC, showed the highest skin permeation and retention of ACV-C_{16}. These results were inconsistent with some studies (Kim et al. 2002; Kirjavainen et al. 1999). We supposed that it might be attributed to the significantly high lipophilic characteristics and encapsulation efficiency of ACV-C_{16}, which played a more important role in enhancing permeability and intradermal retention of ACV-C_{16} than that of cholesterol. The detailed electron-microscopic investigations of the alterations of the stratum corneum bilayer structures coupled with autoradiographic studies would be necessary to further elucidate the mechanism of liposomal action.

2.5. Effects of physicochemical characteristics of ACV-C_{16}

We recently demonstrated that liposomally encapsulated lipophilic ACV-C_{16} could intercalate in the bilayer structure of the lipids in liposomes (Liu et al. 2000). In spite of no measurable amount of ACV-C_{16} being found in the aqueous phase of the liposome preparations, cumulative ACV-C_{16} was present in the receptor buffer solution. These results further supported the hypothesis that the ACV-C_{16} intercalated in the liposomal bilayers might initially be released into the skin and then transfer from skin to the receptor compartment, i.e. the partition of ACV-C_{16} might not between liposomal lipid and aqueous solution but between skin and its surroundings. We thought lipid compositions of liposomes might serve as organic solvents for the solubilization of poorly soluble drugs; as a result, drugs that possess higher lipophilic characteristics were more favorable in dermal delivery of liposomes leading to higher drug concentrations within the skin.

The present *in vitro* study demonstrated that liposomes could increase intradermal concentration and thus facilitated the transdermal efficiency of a water insoluble drug carried by the liposomes. The partitions of ACV-C_{16} and liposomal lipids from viable skin to adjacent tissues might be higher than that observed from *in vitro* study owing to the more lipophilic characteristics of tissue (*in vivo*) than receiver content (*in vitro*) on the receptor side. However, system absorption of a whole liposome vesicle from externally applied liposome preparations would not take place unless the vesicle was small enough and could pass without disintegration through the intercellular junctions of the skin and tissues.

The pronounced higher skin retention of ACV-C_{16} from both positive and skin lipid liposomes implied that the skin-formulation interactions were not a mere absorption, which was favorable for positive liposomes, but that a molecular mixing of the liposomal bilayers with those of stratum corneum bilayers, which was favorable for skin lipid liposomes, might take place.

In conclusion, the liposomal dosage form was superior to the ointment form in terms of topical drug delivery. Lipid compositions of liposomes showed more pronounced influence than charge of liposomes to skin permeation and intradermal retention of ACV-C_{16}, but the phase transition temperature of the lipid was not a critical factor. Skin-lipid liposomed greatly enhanced skin permeation and intradermal retention of ACV-C_{16}.

3. Experimental

3.1. Materials and instruments

Bovine brain ceramide (CA), cholesterol (CH), cholesteryl sulfate (CS), egg lecithin (PC), palmitic acid (PA), stearylamine (SA) and phosphatidylserine (PS) were purchased from Sigma Chemical Co. (St Louis, MO). Acyclovir amorphous powder and ACV-C_{16} ointment (0.5%, w/w) were generous gifts from Keyi Pharmaceutical Co. (Hubei, China). Phosphate buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 12\,H_2O$ and 1.4 mM KH_2PO_4; pH 7.4; 37 °C) and all other products and reagents were of analytical grade. Double-distilled water was always used.

3.2. Animals

The hairless rats were obtained from Wuhan Institute of Biological Products. The institutional Animal Ethical Committee of Wuhan General Hospital approved the experimental protocol for all the studies. The animals were housed under controlled environmental conditions (general food and water were freely available) and treated according to the Guiding Principles for the Administration and Use of Laboratory Animals authorized by the Ministry of Health of China.

3.3. Preparation of ACV-C_{16} liposomes

Liposomes containing ACV-C_{16} 0.5% (W/V) and lipid compositions 20 mg ml^{-1} were prepared (Liu et al. 2002). The liposomal lipid formulations of the tested samples were shown in Table 1. The aqueous phase of liposomes was PBS at pH 7.4 to maintain most ACV-C_{16} in its uncharged form ($pK_a = 7.26$). Appropriate amounts of the lipid mixtures containing ACV-C_{16} were dissolved in 20 ml of chloroform. Five ml of PBS were added to yield a suspension after brief sonication. The organic solvents and a small amount of water were then removed under nitrogen at 45 °C using the RE-52A roto-evaporator (Yarong Biochemical Instruments Co.). When the liposomal suspension became very viscous, an amount of PBS, equivalent to that removed, was reintroduced into the viscous suspension. Traces of solvent that remained in the resultant dispersion were removed until all foaming ceased and then shaken on a vortex mixer to produce large unilamellar vesicles (LUV).

3.4. Determination of physical characteristics of liposomes

One milliliter of liposomal system was diluted about 10 times with double-distilled water and determined using 90 PALS Zeta Potential Analyzer (Brookhaven Instruments Co.). Data of physical characteristics of lipo-

somes (Zeta potential, mean diameter, polydispersity and pH) were obtained under the following measurement parameters: temperature, 25 °C; wavelength, 635 nm; angel, 90°.

3.5. Diffusion experiments

Hairless rat skin was always used. The rats weighing 240-300 g were sacrificed by ether inhalation. The abdominal skin was excised to a size adequate for permeation studies. Diffusion experiments were carried out in the static Franz-type diffusion cells for 48 h, at 37 °C, under continuous stirring, using 0.5% ointment (1 g, w/w) and 0.5% liposome suspensions (1 ml, w/v). The exact cell volumes (15.0 ml) and surface areas (4.91 cm^2) have been taken into account when analyzing permeation data. In the receptor compartment was PBS/isopropanol (1/1, v/v, pH 7.4), in order to ensure pseudo-sink conditions. 0.01% gentamycin was added to the receiver content to prevent skin deterioration during the experiment period.
Before each experiment the system was allowed to equilibrate for 1 h. 500 µl samples were drawn from the receptor compartment and an equivalent amount of fresh PBS/isopropanol (1/1, v/v, pH 7.4) was added each time to maintain a constant volume in the receiver compartment at 1, 2, 4, 8, 10, 12, 24, 36 and 48 h. Dilution of the receiver medium was taken into account when processing the permeation data.

3.6. Analytical methods

The concentration of ACV-C_{16} in the receptor compartment was determined at 257 ± 1 nm using a UV-visible spectroanalyzer (Shimadzu UV-260, Japan). This method followed that of Chen et al. (2000). The cumulative amount of ACV-C_{16} that permeated through the membrane after the nth sampling (Q_n) was calculated by eq. (1):

$$Q_n = C_n V + \sum_{i=1}^{n-1} V_s C_i \quad (1)$$

Where C_i and C_n are the various measured concentrations from 1 to n, V is the volume of the solution in the receptor compartment and V_s is the sampling volume. Fluxes (J) were determined from the slope of the cumulative amount of penetrated ACV-C_{16} versus time, and permeability coefficient (P) was calculated according to eq. (2) based on the fact that drug concentration in the receptor compartment was negligible compared with that in the donor compartment (C_d).

$$P = J/C_d \quad (2)$$

3.7. Statistical analysis

The results are expressed as the mean ± standard deviation of 4 animals. Statistical analysis was carried out using Student's unpaired t-test and one-way analysis of variance (ANOVA). Significance was taken as $P < 0.05$ and $P < 0.01$.

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