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The influence of particle size of liposomes on the deposition of drug into skin

J. du Plessis [a], C. Ramachandran [b], N. Weiner [b] and D.G. Müller [a]

[a] *Department of Pharmaceutics, Potchefstroom University for CHE, Potchefstroom (South Africa) and* [b] *College of Pharmacy, University of Michigan, MI (USA)*

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Summary

The effect of particle size of liposomes on the deposition of drugs into the strata of skin was evaluated using hairless mice, hamster and pig skin. In vitro diffusion studies were performed in an attempt to find an optimum formulation for topical drug delivery as well as to try to explain the mechanism of topical drug delivery by liposomes. The results indicate that an optimum particle size for optimal drug delivery exists. The study proved that the follicular route play an important role in determining the kinetics of drug transfer from liposomes into the skin.

Introduction

Mezei and Gulasekharam (1980, 1982), Singh and Mezei (1983) and Schaeffer et al. (1982) were the first to report the potential use of liposomes in topical applications for the skin and eyes. Liposomes have proven to be useful in the systemic treatment of skin diseases. Drugs can be targeted to the site of the infection and side-effects can be kept to a minimum by the prevention of systemic absorption of the drug. Several factors such as lamellarity, lipid composition, charge on the liposomal surface and the total lipid concentration have been proven to influence drug deposition into the deeper skin strata (Weiner et al., 1989).

Egbaria et al. (1990) studied the extent of uptake of cyclosporin-A (CSA) after the topical application of 'skin lipid' liposomes and phospholipid liposomes. High levels of CSA was found in the stratum corneum after CSA was topically applied in the form of liposomes.

There has been much speculation concerning the mechanism by which liposomes deliver drugs into the skin. Mezei and Gulaksekharam (1980) suggested that liposomes pass intact through the lipid rich outer layer of the skin to the dermis, where they become localised. Ganesan et al. (1984) as well as Ho et al. (1986) criticised this theory on the basis that the size of liposomes would not allow diffusion of liposomes through the densely packed outer layers of the skin to arrive at the deeper stratum corneum, living epi-

Correspondence to: J. du Plessis, Department of Pharmaceutics, Potchefstroom University for CHE, Potchefstroom, South Africa.

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dermis or the dermis intact. Egbaria and Weiner (1991) suggested that a molecular mixing of the liposomal bilayers and those of the stratum corneum takes place. There have also been indications that the follicular pathway contributes to the liposomal delivery of drugs into the deeper skin strata (Du Plessis et al., 1992).

It was assumed that if it is possible for liposomes to cross the stratum corneum intact, a decrease in the particle size of the liposomes would result in an increase of the amount of drug found in the deeper skin strata. The aim of this study therefore was to investigate the effect of particle size of liposomes on the deposition of drugs into the strata of the skin by using in vitro diffusion studies in an attempt to find the optimum formulation for topical drug delivery, as well as to try to explain the mechanism of topical drug delivery by liposomes.

Materials and Methods

Materials

Cholesterol (CH), cholesteryl sulphate (CS), bovine brain ceramides (CM), palmitic acid (PA), stearylamine (SA) and Hepes free acid were obtained from Sigma (St. Louis, MO). Hepes free acid (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]) has a buffer capacity of 6.8–8.2 and a pK_a of 7.5 at 25°C. Egg lecithin (PC) was obtained from Avanti Polar Lipids (Birmingham, AL) and α-tocopherol (α-T) from Eastman Kodak (Rochester, NY). All other chemicals were of analytical grade and the water used was double distilled, deionized and filtered with a Milli-Q system (Millipore Corp., Bedford, MA). CSA was obtained from the School of Dermatology, University of Michigan and the radiolabelled CSA was obtained from Amersham (U.K.).

Methods

Liposome formulations Large unilamellar vesicles containing phosphatidylcholine/ cholesterol/cholesteryl sulphate (PC/CH/CS) at a molar ratio of 1:0.5:0.1 were prepared by a modification of the reverse-phase evaporation method (REV) of Szoka and Papahadjopoulos (1978). Appropriate amounts of the lipid mixtures were dissolved in 10 ml of a chloroform-methanol mixture (2:1; v/v). 5 ml of 0.05 M Hepes buffer (pH 7.4) and enough additional methanol (up to 1.5 ml) were added to yield a clear solution after brief sonication. The organic solvents and a small amount of water were then removed under nitrogen at 45°C. Solvent removal was continued until all foaming ceased. The liposomes contained 1.16 mg/ml CSA and trace amounts of radiolabelled CSA and CS were included. The CSA as well as the radiolabelled markers were dissolved in the organic solvent before the preparation of the liposomes. Liposomes with different particle sizes were obtained by homogenisation of the liposomes with a Microfluidizer (Microfluidizer Model M110, Microfluidics Corp., Newton, MA) at constant pressures by varying the time and length of homogenisation. The encapsulated fractions (fractions 2 and 3) of the liposomes were collected by exclusion chromatography with a Sephadex G-75 column (Sigma Chemical Co., St. Louis, MO). The collected fractions were dehydrated under vacuum to the appropriate volume so that the final liposome preparation contained 25 mg/ml of lipid. This lipid concentration was chosen because it was found that the largest amount of CSA could be encapsulated using this concentration. The mean volume diameter of the liposomes was determined with the Nicomp Submicron Particle sizer (Model 370, Pacific Scientific Instrument Division, Silver Spring, MD).

Deposition experiments Full thickness hairless mouse skin and hamster skin were excised from fresh carcasses and subcutaneous fat was carefully removed using a scalpel. Pig skin was obtained from a local abattoir and cleaned of any subcutaneous fat. The skin sections were mounted on Franz diffusion cells with nominal surface areas of 2 cm^2 and receiver compartments with 7 ml capacities (Crown Glass, Somerville, NJ). The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by a 0.05 M isotonic Hepes buffer. The receiver solution was stirred continuously, using a small Teflon covered magnet. Care was exercised to remove any air bubbles between the underside of the skin and solution in the receiver compart-

ment. The temperature of the receiver was maintained at 37°C. Following mounting of the section of skin, 200 μl of the test formulation (1.16 mg/ml CSA) was applied to the skin. A minimum of three cells were used for each formulation, using sections of skin from different skin specimens for each formulation. Pig, hamster and hairless mouse skin were used in the experiments in order to determine the contribution, if any, of the follicular route on the deposition of liposomal lipids. The hairless mouse skin is almost devoid of any follicles, whereas the pig skin has very well distinguished follicles. All experiments were carried out with non-occluded donor compartments. After 24 h, the experiments were stopped and the diffusion set-up was dismantled for assay of radiolabelled lipids.

Assay of radiolabelled markers Upon dismantling, the donor compartment of the cell was carefully rinsed five times with 0.5 ml buffer, after which the skin was removed. The skin was also rinsed twice with 3 ml of buffer. This washing procedure was found to be sufficient to remove more than 99% of the formulation when determined at time zero (washing of the skin directly after the application of the formulation).

All washings were collected and assayed for radiolabel. A common procedure for the determination of phospholipid levels in the stratum corneum involves stripping of the skin. The skin patch was mounted on a board and a piece of adhesive tape (Scotch Magic Tape, 810, 3M Commercial Office Supply Division, St. Paul, MN), 1.9 cm wide and about 6 cm long, was used to strip the skin. The tape was of sufficient size to cover the full area of the skin which was in contact with the formulation. The stripping procedure was repeated until the stratum corneum was completely removed, as determined by transepidermal water loss and visual observation (Tsai et al., 1991). It was determined that nine such strippings were necessary for mouse skin and 18 strippings for pig skin to ensure removal of the stratum corneum. Each strip was analysed separately for radiolabelled lipid. The remaining skin, as well as the receiver compartment solution, were also assayed for lipid content. Assays of the donor, skin rinses and receiver solutions were carried out after addition of about 15 ml of the scintillation fluid Ecolite$^+$ (ICN Biomedical, Inc., Irvine, CA) to every sample. The tape strippings and remaining skin were assayed as follows: each sample was placed in a combustion-cone and burnt in a tissue oxidiser (Model 306 Packard oxidiser, Packard Instrument Co., Downers Grove, IL). The separated radionuclides were assayed using a scintillation counter.

Results

The amounts of CSA delivered from liposomes from different particle sizes into the strata of the skin are shown in Table 1. The amounts of CS delivered by the same liposomal formulation is shown in Table 2. The amounts of CSA and CS adhering to the stratum corneum surface were determined by analysis of the first two tape strippings. The amounts of CSA and CS penetrating the deeper stratum corneum were determined by the analysis of tape strippings 3–9 for hairless mouse, 3–15 for hamster and 3–20 for pig skin. The amounts of CSA and CS in the deeper skin strata were determined by analysis of the remainder of the full thickness skin.

Discussion

Prior to this study, it was expected that smaller particle sizes of liposomes would result in an increase in drug deposition if liposomes cross the stratum corneum intact. However, the results of this study (Table 1) clearly showed that the smaller liposomal particle sizes (0.06 μm) did not result in higher CSA levels in the deeper skin strata of any of the skin species used. It is thus clear that the mechanism of action for topical liposomal drug delivery does not involve the passage of intact liposomes.

The intermediate particle size of 0.3 μm resulted in both the highest reservoir in the deeper skin strata (except in pig skin) as well as the highest drug concentration in the receiver. It seems as if there might be an optimum particle size for optimal drug delivery. However, a more

TABLE 1

The amounts of cyclosporin A (CSA) (μg) delivered from PC / CH / CS REV liposomes with three different particle sizes into the different strata of hairless mouse, hamster and pig skin

Compartment	Hairless mouse			Hamster skin			Pig skin		
	(particle size)			(particle size)			(particle size)		
	0.06 μm	0.3 μm	0.6 μm	0.06 μm	0.3 μm	0.6 μm	0.06 μm	0.3 μm	0.6 μm
Total donor	41.48 ± 11.1	78.89 ± 8.4	49.88 ± 3.4	15.12 ± 2.7	26.50 ± 2.3	23.38 ± 2.7	27.93 ± 1.1	31.04 ± 0.9	44.82 ± 7.0
Surface stratum corneum	167.48 ± 7.2	111.10 ± 2.0	122.59 ± 9.2	49.83 ± 5.6	53.50 ± 2.3	58.84 ± 2.9	90.32 ± 13.5	64.94 ± 23.6	77.19 ± 7.7
Deeper stratum corneum	11.67 ± 2.1	24.94 ± 3.3	49.81 ± 0.8	157.81 ± 18.4	131.40 ± 7.3	131.40 ± 2.7	93.17 ± 9.5	92.45 ± 7.9	63.70 ± 3.2
Deeper skin strata	7.17 ± 1.8	10.60 ± 0.4	6.29 ± 1.0	5.64 ± 1.9	9.93 ± 3.9	8.96 ± 2.4	9.93 ± 2.7	34.68 ± 7.0	18.49 ± 3.9
Total skin	186.32 ± 10.5	146.64 ± 4.8	178.69 ± 9.4	213.28 ± 6.9	194.83 ± 2.6	199.2 ± 2.8	193.42 ± 1.7	192.07 ± 1.3	160.38 ± 10.6
Receiver	4.20 ± 0.8	6.38 ± 3.6	3.43 ± 0.8	3.60 ± 0.2	10.67 ± 2.5	9.42 ± 1.6	10.65 ± 2.5	8.89 ± 3.6	26.80 ± 5.4

extensive study should be undertaken to find the exact optimum particle size. The results, at the least, suggest that topical drug delivery is influenced by the particle size of the vesicles of the liposomal suspension.

Considering the amount of CSA in the receiver, both hairless mouse and hamster skin showed the highest amounts of CSA when the intermediate (0.3 μm) particle size was used (6.38 and 10.67 μm, respectively). However, the amount in the receiver in the case of pig skin was not markedly higher compared to the amounts found in hairless mouse or hamster skin. Due to the lipophilic nature of both CSA and the pig skin, the release of CSA to the receiver may be retarded.

The amounts of drug deposited in the deeper skin strata of pig skin (34.68 μg at particle size of 0.3 μm) were much higher than those in hairless mouse (10.80 μg). One should expect that the large amount of follicles in hamster skin would lead to a higher concentration of CSA in hamster skin when compared to hairless mouse skin. Surprisingly, hamster skin did not show a higher concentration of CSA in the deeper skin strata compared to hairless mouse skin as was found with γ-interferon (Du Plessis et al., 1992) and transforming growth factor α (Du Plessis, 1992). CSA is a very lipophilic molecule as opposed to γ-interferon and transforming growth factor α which are both very hydrophilic molecules. It seems as if the lipophilic drug prefers the lipoidal pathway for skin penetration and that the presence of follicles does not play such an important role in the topical delivery of lipophilic drugs. However, the follicular pathway should not be ignored, as indicated by the results in Table 1 for pig skin.

Ritschel et al. (1991) proposed a mechanism by which an intracutaneous depot is formed by using the sorption promoter Transcutol® as solvent in topical drug delivery. The stratum corneum is a heterogeneous structure consisting of protein bricks and lipid mortar (Elias, 1983). According to Elias et al. (1981) and Williams and Elias (1989), the intercellular lipids are important in controlling the percutaneous absorption. They found that Transcutol® caused swelling of intercellular spaces and accumulation of foreign material outside the cell membrane. It seems as if Transcutol® causes swelling of the intercellular lipids without altering the multiple bilayer structure of the stratum corneum. These swollen lipids may cause accumulation of the drug and thereby form and intracutaneous depot. In the case of liposomes the phospholipids may mix with the intercellular lipids and thereby cause the same swelling effect to form an intracutaneous depot. However, further evaluation including electron-microscopy studies is necessary to confirm this hypothesis.

An examination of Table 2 reveals that the particle size of the vesicles had the same effect on the deposition of liposomal lipid (CS) which it

TABLE 2

The amounts of cholesteryl sulphate (CS) (μg) delivered from PC / CH / CS REV liposomes with three different particle sizes into the different strata of hairless mouse, hamster and pig skin

Compartment	Hairless mouse			Hamster skin			Pig skin		
	(particle size)			(particle size)			(particle size)		
	0.06 μm	0.3 μm	0.6 μm	0.06 μm	0.3 μm	0.6 μm	0.06 μm	0.3 μm	0.6 μm
Total donor	20.86 ± 8.4	36.79 ± 4.3	19.02 ± 4.1	22.5 ± 7.3	11.18 ± 0.2	10.27 ± 0.8	15.14 ± 4.0	18.11 ± 3.2	23.03 ± 2.3
Surface stratum corneum	77.30 ± 8.4	58.34 ± 2.5	62.24 ± 10.0	33.04 ± 9.8	36.9 ± 4.5	40.96 ± 0.5	50.49 ± 8.5	46.92 ± 2.3	43.85 ± 2.2
Deeper stratum corneum	1.41 ± 0.3	2.8 ± 1.9	17.68 ± 4.0	43.43 ± 2.4	49.75 ± 5.9	47.62 ± 1.6	32.89 ± 2.2	32.40 ± 0.1	29.46 ± 0.3
Deeper skin strata	0.16 ± 0.0	1.14 ± 0.3	0.84 ± 0.2	0.81 ± 0.3	1.69 ± 0.4	0.87 ± 0.2	1.01 ± 0.3	2.00 ± 0.2	12.69 ± 0.4
Total skin	78.87 ± 8.1	62.28 ± 3.9	80.76 ± 3.9	77.28 ± 3.4	88.34 ± 1.0	89.35 ± 1.9	84.39 ± 5.1	81.32 ± 2.2	76.0 ± 2.3
Receiver	0.27 ± 0.0	0.93 ± 0.1	0.22 ± 7.9	0.22 ± 0.0	0.48 ± 0.0	0.38 ± 0.1	0.47 ± 0.2	0.57 ± 0.2	0.97 ± 0.1

had on CSA delivery in hairless mouse skin. However, considering the standard deviation, particle size did not have any influence on the deposition of CS in hamster or pig skin. Once again, the largest amount of CS was deposited in pig skin, where the most pronounced follicular pathway is present.

It is interesting to note that the smallest particle size (0.06 μm) resulted in the highest amounts of CSA and CS in the surface stratum corneum in hairless mouse as well as pig skin (Tables 1 and 2).

It thus seems as if the size of the liposomes regulates the location of the depot effect of lipophilic drugs in the skin. Until a suitable method of analysis is available, by which both the skin and the liposomes can be visualised as well as distinguished, speculation about the mechanism of topical liposomal drug deposition will continue.

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