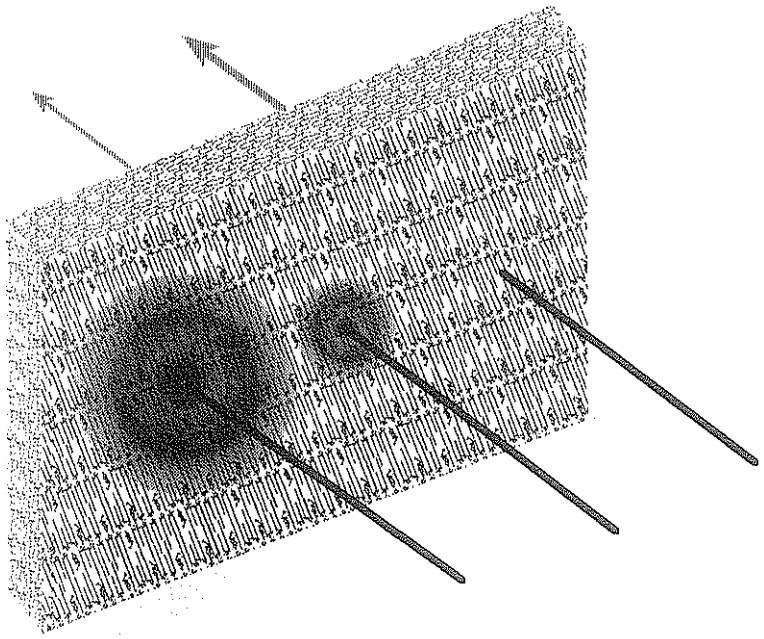
Perspectives in Percutaneous Penetration

Volume 5b



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this volume is dedicated to the memory of

Valerie Jane James

1955-1998

FOREWORD

The Fifth PPP Conference was once again organised in La Grande Motte, France, in April 1997. The continuation of a high level of interest in the area was reinforced by the large number of contributed presentations which continued the tradition set at the previous four meetings. In recognition of the evolution of the scope of this conference over the years the title of "PPP" has now been redefined in terms of the very wide range of "Perspectives" presented, rather than only the original more circumscribed aspect of "Prediction".

The purpose of the PPP Conference is to provide a forum for the presentation and discussion of all the latest information and technology in the penetration of exogenous compounds through the skin. The meetings are therefore particularly relevant to all scientists involved in research, development, risk assessment and regulatory affairs relating to the dermal effects of molecules within the agrochemical, cosmetic and pharmaceutical fields within industrial, academic and governmental domains.

This volume contains a collection of selected papers covering all aspects of skin permeation from a wide range of authors including both invited speakers and other contributors to the meeting.

The Sixth International PPP Conference will take place in September 1998 in Leiden, the Netherlands.

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PERCUTANEOUS ABSORPTION OF LIPOSOMES FORMED BY LIPIDS MODELLING THE STRATUM CORNEUM

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INTRODUCTION

The mimetic character of liposomes in relation to the organized lipid structures of the stratum corneum (SC) may provide a suitable strategy for achieving an accurate vehiculization of a particular compound during the first stage of its percutaneous absorption. Recent work has demonstrated a small but significantly enhanced penetration of phosphat-dylcholine liposomes for the release of sodium fluorescein using the stripping technique'. In the same line, the influence of the liposome permeability characteristics and the lipophilic/ hydrophilic character of the probe on percutaneous absorption has been studied[2].Lipid model mixtures from SC lipids have been reported to form stable liposomes[3,4] and their physico-chemical properties have been established depending on the different lipid composition[5,6].

The present work deals with "in vivo" stripping technique to evaluate the percutaneous absorption of sodium fiuorescein vehiculized in different liposome preparations with lipid compositions related to the ones present in the SC. Different lipid compositions modelling the stratum corneum have been considered to form liposomes, studying their vesicle size distribution, encapsulation efficiency, lipid analysis and microscopic analysis of the formed vesicles. The main aim of the present work is to study the percutaneous penetration of such liposomes in relation with a conventional phospholipid liposome to shed a light on the molecular mechanism of liposome penetration enhancement, that could be very useful in designing specific liposomal formulations for topical applications.

MATERIALS

Reagent grade orgenic solvents, ceramides type III (Cer) and fluorecein sodium salt (NaFl) were from Sigma (USA). Palmitic acid (PA) and cholesterol (Chol) from Fluka (Switzerland). Cholesteryl sulfate (Chol-S) was prepared by reaction of cholesterol with excess cholorosulfonic acid in pyridine and purified chromatographically. Lipid S-100, whose main component is soy phosphatidylcholine (>95%) was from Lipoid (Germany) Boric acid, potassium chloride, sodium hydroxide, sodium chloride and hydrochloric acid, were from Merck (Germany).

METHODS

Preparation of liposomes

Liposomes with different lipid compositions modelling the stratum corneum were prepared (Table 1). Furthermore, phospholipid liposomes were also made with S-100. Individual lipids were dissolved in chloroform/methanol 1:1 (v/v) and appropiate volums were combined to obtained several mixtures, as shown in Table 1.

Table 1 **Composition of** lipid mixtures **(wt %)**

Mixture	Cer	Chol	PA	Chol-S
A	40.0	25.0	25.0	10.0
B	35.0	25.0	23.0	17.0
C	45.0	25.0	27.0	3.0
D	25.0	25.0	25.0	25.0

Lipid mixtures were evaporated to dryness in a round bottom flask in a rotary evaporator under reduced pressure at 60°C to form a thin film on the flask. The film was hydrated with saline solution of NaFl (155mM NaCl, 2.66mM NaFl) to give a final concentration of lipid of 10mg/mL and NaFl of 1mg/mL. Multilameliar vesicle liposomes (MLV) were formed by constant vortexing for 5 min. and sonication for 5 min. in a bath sonicator (514 ECT Selecta). MLV liposomes were downsized to form oligolamellar vesicles by sonication[7] or extrusion[8]. Sonication was performed at 80°C (temperature higher than PTT of lipids involved) until the suspensions became clear (≈15min), and were then annealed at 80ºC for 30 min, in the case of the SC liposomes. Extrusion was performed through polycarbonate membrane filters of variable pore size under N_2 pressures of up $55x10^5$ Nm^{-2} at 37°C in an Extruder device (Lipex Biomembranes, Canada), in the case of phospholipid vesicles. Liposomes containing the encapsulated probe were purified twice, to separate the encapsulated probe from free fluorescein, by centrifugation in a Ti type rotor of a Beckmann L-8-60M uitracentrifuge by adding the same volume of saline solution, oentrifuging (7,000g) for 30min at 7°C and discarding the same supernatant volume.

Characterizaiion of liposomes

Deterrnination of vesicle size distribution has been made at 25°C by dynamic light scattering employing an Autosizer 1l c photon correlation spectrorneter (Malvern, UK).

Quantitative lipid analysis of liposomes made up with phospholipid S-100 was performed by the Steward-Marshall method[9] and the analysis of liposomes made up with SC lipids was obtained by thin -layer chromatography coupled to a flame ionization detector (TLC/FID) (Iatroscan MK-5, Iatron Laboratory, Inc., Tokyo, Japan)[10] following the methodology previously described[11]. The NaFl content was spectrophotometricaliy determined with a spectrophotometer V-200 Hitachi (Japan) at 20°C by adding 1.5ml of saline solution and 1.5ml of SDS-borate buffer (4%, pH=8.9) to 100ml of liposomes to break the liposomes and release the NaFl (λ=493nm). The encapsulation efficiency was then determined as a percentage of probe NaFl in the purified sample related to the initial amount. Electron microscopy visualization of some liposome formulations was performed in a Hitachi H-600AB transmission electron microscope operatlng at 75kv. Freeze-fracture, etching and coating were carried out at -110°C in a vacuum >$5x10^{-7}$ mbar using a BALTEC type BAF-060 (Balzers AG, Lichtenstein).

Penetration studies

Penetration behaviour was studied in vivo by the stripping method[12] applying the same concentration of NaFl in all formulations(1mg/mL). Topical application assays and the 15 stratum corneum strips were carried out as previously described'. The extraction of the NaFl content in the different strips was done with 5mL of methanol shaking for 1h and then adding 5mL of borate buffer (pH=9.0) to increase the fluorescence. NaFl fluorescence of these samples was measured spectrofluorimetrically[1]

RESULTS AND DISCUSSION

Stable liposomes have been reported to be able to be formed with lipids modelling the stratum corneum when hidrated at 80°C[3-6]. Wertz and co-workers[3] prepared liposomes from lipid mixtures containing only ceramides (40%), cholesterol (25%), free fatty acids (25%) and cholesteryl sulfate (10%), approximating the Composition of stratum corneurn lipids; therefore we chose this composition A (Table 1) as the most similar to that of the stratum corneum. Physichochemical properties of liposomes formed with related compositions have also been studied[5,6]. Since increasing concentrations of cerarnides, palmitic acid and cholesterol (or low cholesteryl sulfate concentrations) results in a modification of these structures with a decrease on the percentage of a fluorescen probe release[6], variations of this composition A have been studied (B, C and D) to determine their possible different behaviour on percutaneous absorption (Table 1).

Since the lipids of the inner pari of the epidermis are mainly made up with phospholipid, liposomes S-100 were also prepared with the aim to compare its behaviour with liposomes made by mixtures of lipids modeling the composition of the stratum corneum.

Preparation of liposomes were performed, at least in duplicate for each mixture, with the aim to obtain vesicles with similar structure and size (unilamellar vesicles of 200nm) with also the same amount of total lipid amount (10mg/mL) and fluorescence probe (1mg/mL). SC liposomes were sonicated at 80°C for different times measuring the size of an aliquot until a particle size distribution of about 200nm was achieved for A,B and C mixtures, eventhough liposome D did not get to this size (Table 2).

Lipid content was quantified by TLC/FID in the case of SC liposomes and with the colorirnetric method of Steward-Marshall in the case of phospholipid liposomes. Encapsulation efficiency was obtained spectrophotometrically after liposomes were purified twice by centrifugation as described in the experimental section. The results obtained are listed in Table 2.

Table 2 **Particle** size distribution, lipid concentration, and encapsulation efficiency of the **different** liposomes

Liposome	Size distribution Size (nm)	P.I.	Lipid Conc. (mg/mL)	Encapsulation efficiency (%)
A	212	0.357	10.2	35.5
B	174	0.358	8.5	37.4
C	228	0.354	4.7	37.9
D	404	0.286	7.8	40.3
S-100	204	0.145	10.1	34.0

It has to be pointed out the similar size obtained for the A,B and C liposomes with a polidispersity index, however, higher to the one obtained for the phospholipid liposome. As we above mentioned, liposomes D were notable to be downsized to the desired size by this methodology. Liposomes B, D and specially C were more difficult to be formed. This is the reason for the poor lipid retrieval of these samples. It seems that the composition more similar to the one present in the stratum corneum A, is the most easier to be formed with a complet retrieval of the total lipid used.

Even though the methodology for liposome purification do not lead to the liposomes completly free of unencapsulated probe, the spectrophoio metrically measurement of these purified samples give an idea of the encapsulation efficiency of the different liposomes, obtaining a higher percentage for the biggest size liposomes D.

Electron microscopy of liposomes A and S-100 were performed to confirm size and structure of the samples (Fig.1). Even the different lipid composition of the vesicles A and S-100, the photomicrographs confirm the size and similar structure of the liposomes formed.

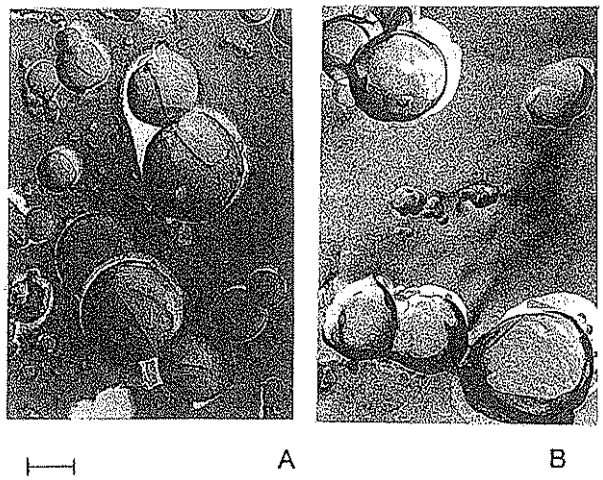


Fig. 1 TEM photomicrograph of liposomes **A (A)** and **S-100** (B) obtained by freeze-fracturing. Bar represents **100** nm.

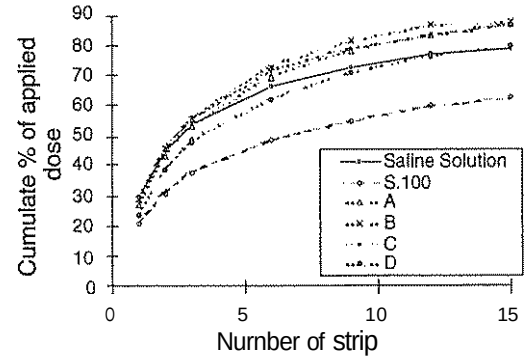
In order to study the different penetration behaviour of these vesicles the stripping method have been applied for the different liposomes (Table 2) and for the same concentration of NaFl in a saline solution (0.9% NaCl). Each assay was done for the six formulations prepared on four volunteers in duplicate. The amount of NaFl recovered in every strip or group of strips expressed in percentage with respect to the total dose applied for the different formulations was determined (Table 3).

Table **3** Recovery % of applied dose in the different strips

Nº Strip	Sal. sol.	S-100	A	B	C	D
1	30.15	20.67	26.94	28.77	29.54	23.79
2	14.98	10.35	16.55	16.98	15.93	15.11
3	8.37	7.09	9.86	10.14	9.74	9.45
4-6	12.61	10.48	16.19	16.97	16.30	14.05
7-9	6.85	6.25	8.93	8.49	7.40	8.64
10-12	3.87	5.08	5.23	5.12	4.46	5.43
13-15	2.41	2.78	3.28	3.09	3.44	3.21
Tot %	79.24	62.70	86.98	89.56	86.81	79.68
SC %	49.09	42.03	60.04	60.79	57.27	55.89

Bearing in mind that the NaFl content in the first strip accounts for the non-penetration content, the higher amount of NaFl content obtained in this first strip when saline solution is applied with respect to liposomes, specially S-100, should be noted. The weakness of the SC barrier effect when liposomes are applied is not very marked for liposomes A, B and C with lipid composition similar to the SC. Furihermore, the total percentages of NaFl anaiyzed by adding all the strips are much lower in the case of S-100 liposome. This result reflects the highest penetration in deeper layers of epidermis, which also highlights the afore-mentioned weak barrier effect. However, the total amount of NaFl in the SC (from the 2nd to the 15th strip) was also calculated to determine the reservoir capacity of this layer. The highest amount of NaFl in SC when liposomes made up with lipids modelling the stratum corneum are topically applied, specially A,B and C, indicates the higher reservoir capacity of this layer when similar lipid composition formulation is applied. In order to follow the penetration profile of NaFl in the different strips, cumulative percentages were calculated and piotted as a function of the strip numbers (Figure 2). This figure shows the similar behaviour of liposomes A, B and C with a total diminution of probe penetration when compare with saline solution and moreover with S-100 liposome in which the penetration is clearly enhanced.

Fig. **2** Penetration of NaFl into stratum corneum **after 30** min



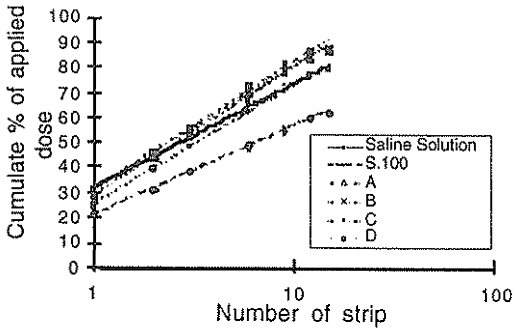
The shape of the curves obtained indicates a logarithmic relationship between the NaFl cumulative values and the different strips. In fact a regression analysis was performed and the functions $y=alog(x)+b$ were obtained, y being the cumulative % of applied dose, x the strip number and a and b the estimated equation coefficients. Parameters a, b and the determination equation coefficient R^2 are given in Tabie 4.

Table 4 Mathematical equation parameters and determination coefficients in different formulations

Formulation	a	b	R^2
Saline sol.	18.20	32.02	0.9933
S-100	15.66	20.56	0.9996
A	22.49	27.94	0.9974
B	22.46	30.32	0.9921
C	21.43	30.82	0.9951
D	20.85	24.54	0.9985

The regression curves and the experimental points are also expressed in Fig.3 logarithmicaily for the sake of clarity.

Fig. 3 Relationship between cumulative % of NaFl in the stratum corneum and the logarithm of the strip number



It shouid be noted the accurately fit of experimental points, having high correiation coefficients in all cases. The values of constant ***b,*** which account for the non-penetration content, are lower in the case of liposome S-100 which impiies again the high penetration enhancement of this formulation. The slopes a, which account for the penetration rate inside the stratum corneum are higher and similar for the four liposomes with lipid composition modeiling the stratum corneum, this implies their higher afinity for this layer.

CONCLUSIONS

These results indicate the compiete different penetration behaviour of liposomes of simiiar size and structure but different lipid composition, leading to a clear enhanced penetration of the vesicles formed with phosphoiipids and a diminution in the case of liposomes formed with SC iipids. It is important to emphasize the high amount of probe found in the stratum corneum, reflected also in a high slope of the logarithmic curves, when SC liposomes are applied. The mimetic character of these structures with those present in stratum corneum could be the reason for increase in reservoir capacity of this layer.

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