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RESEARCH PAPER

Transfersomes—A Novel Vesicular Carrier for Enhanced Transdermal Delivery: Development, Characterization, and Performance Evaluation

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ABSTRACT

This work describes the use of a novel vesicular drug carrier system called transfersomes, which is composed of phospholipid, surfactant, and water for enhanced transdermal delivery. The transfersomal system was much more efficient at delivering a low and high molecular weight drug to the skin in terms of quantity and depth. In the present study transfersomes and liposomes were prepared by using dexamethasone as a model drug. The system was evaluated in vitro for vesicle shape and size, entrapment efficiency, degree of deformability, number of vesicles per cubic mm, and drug diffusion across the artificial membrane and rat skin. The effects of surfactant type, composition, charge, and concentration of surfactant were studied. The in vivo performance of selected formulation was evaluated by using a carrageenan-induced rat paw edema model. Fluorescence microscopy by using rhodamine-123 and 6-carboxyfluorescein as fluorescence probe was performed. The stability study was performed at 4°C and 37°C. An in vitro drug release study has shown a nearly zero order release of drug and no lag phase. The absence of lag phase in comparison to liposomes and ointment is attributed to the greater deformability, which may account for better skin permeability of transfersomes. In vivo studies of transfersomes showed better antiedema activity in comparison to liposomes and ointment, indicating better permeation through the penetration barrier of the skin. This was further confirmed through a fluorescence microscopy study. Finally, it may be concluded from the study that complex lipid molecules, transfersomes, can increase the transdermal flux, prolong the release, and improve the site specificity of bioactive molecules.

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INTRODUCTION

The percutaneous route for drug administration has many advantages over other pathways, including avoidance of first pass effect, delivering drugs continuously, having fewer side effects, and improving patient compliance.[1] However, the barrier nature of skin inhibits the penetration of most drugs. The use of lipid vesicles in a delivery system for skin treatment has attracted increasing attention in recent years, but it remains controversial. Most relevant reports cite the localization effect of liposomes[2–4] with transport processes reported in a few cases, depending on the formulation. To overcome the above problem, a novel type of highly deformable lipid vesicles called transfersomes has been reported recently to penetrate intact skin, if applied nonocclusively[5,6] Transfersomes™ have been defined as specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. Accordingly, transfersomes resemble lipid vesicles, liposomes, in morphology but, functionally, transfersomes are sufficiently deformable to penetrate pores much smaller than their own size. They are (quasi) metastable, which makes the vesicle membrane ultraflexible, and, thus, the vesicles are highly deformable. It is chiefly the unusually strong membrane adaptability that allows the transfersomes vesicles to accommodate to a confining pore and thus trespass such a pore. Typical transfersomes are, therefore, characterized by at least one order of magnitude more elastic membrane than that of conventional lipid vesicles, liposomes. In order to change liposomes into transfersomes, one can incorporate one or more edge-active substance(s) into the vesicular membrane, surfactants were suggested as examples of such edge-activators.[7–9] Another specific difference between transfersomes and liposomes is the higher hydrophilicity of the former, which allows transfersome membrane to swell more than conventional lipid vesicle bilayers. Higher membrane hydrophilicity and flexibility both help transfersomes to avoid aggregation and fusion, which are observed with liposomes exposed to an osmotic stress.[10,11]

The aim of the present study was to refine the formulation of transfersomes for delivery of a model lipophilic drug, dexamethasone, and to evaluate Span-80, Tween-80, and sodium deoxycholate as edge-activators.

MATERIALS AND METHODS

Materials

Soyaphosphatidyl choline (PC), cholesterol, sodium deoxycholate, sephadex-G-50, stearyl amine, dicetyl phosphate, phosphotungastic acid, polyethylene glycol (PEG)-4000, PEG-400, rhodamine-123, 6-carboxyfluorescein, carrageenan, and triton X-100 were purchased from Sigma Chemicals, Span-80 and Tween-80 were purchased from Loba Chemie, India. Ethanol, isopropyl alcohol, butanol, xylene, and chloroform were procured from E. Merck, India. Cellophane membrane (molecular weight cutoff, 12,000 to 14,000) was procured from HIMEDIA, India Ltd. All reagents were of analytical reagent (AR) grade. Double distilled water was used for all experiments.

Preparation of Transfersomes and Liposomes

The transfersomes were prepared by the conventional rotary evaporation sonication method described by Cevc et al.[12] and El. Maghraby et al.[13] Precisely, phospholipid, surfactant, and the drug were taken in a clean, dry, round-bottom flask, and the lipid mixture was dissolved in methanol or in 2:1 v/v chloroform:methanol. The organic solvent was removed by rotary evaporation (Rotary evaporator; York Scientific Ltd., India) under reduced pressure at 40°C. Final traces of solvents were removed under vacuum overnight. The deposited lipid film was hydrated with 7% v/v ethanol by rotation at 60 rev min^{-1} for 1 hr at room temperature. The resulting vesicles were swollen for 2 hr at room temperature to get large multilamellar vesicles (LMLV). To prepare smaller vesicles, LMLVs were probe sonicated at 4°C for 20 min at 40% output frequency (at 40 W) (Titanium probe, Ultrasonicator; Imeco Ultrasonics, India). The sonicated vesicles were extruded through a sandwich of 200 and 450 nm polycarbonate membranes (Millipore). The final lipid and drug concentration in all transfersomal formulation was 5% w/v and 0.05% w/v, respectively.

The liposomes (phosphatidyl choline:cholesterol; 7:3) that act as a control in the present study were prepared by the same method as described above. The final lipid and drug concentration was 5% w/v and 0.05% w/v, respectively.

Vesicles Shape and Type

Transfersomes vesicles were visualized by using a Philips transmission electron microscope (TEM), with an accelerating voltage of 100 kV. A drop of the sample was placed onto a carbon-coated copper grid to leave a thin film on the grid. Before the film dried on the grid, it was negatively stained with 1% phosphotungastic acid (PTA). A drop of the staining solution was added onto the film, and the excess of the solution was drained off with a filter paper. The grid was allowed to air dry thoroughly and samples were viewed on a transmission electron microscope.[14]

Transfersomes vesicles (without sonication) also were visualized by phase-contrast microscopy by using an optical microscope (Leica, DMLB, Germany). A thin film of transfersomes was spread on a slide and, after placing coverslip, was observed under the optical microscope.

Vesicles Size and Size Distribution

The vesicles size and size distribution were determined by dynamic light scattering method (DLS), using a computerized inspection system (Malvern Zetamaster, ZEM 5002, Malvern, U.K.). For vesicles size measurement, vesicular suspension was mixed with the appropriate medium (7% v/v ethanol) and the measurements, were conducted in triplicate.[15]

Entrapment Efficiency

The entrapment efficiency was determined after separation of the unentrapped drug by the minicolumn centrifugation method.[16] Sephadex G-50 was swollen in distilled water at room temperature, with occasional shaking, for at least 5 hr, after which the gel was formed and stored at 4°C. To prepare the minicolumn, whatman paper pads were placed at the bottom of the barrels of 1.0-mL syringes, which were filled with the gel. Excess water was removed by centrifugation at 3000 rev min^{-1} or 3 min, and a 200-μL transfersomes suspension was applied dropwise to the center of the column. Followed by centrifugation, as before, and collection of vesicles, distilled water was added to the minicolumn, and centrifugation was repeated. Transfersomes (depending on their type and size) can be recovered from the first or the second stage of centrifugation.[15,17] Here two stages were necessary to recover the vesicles. No free drug remained (tested by its absence in the centrifugate after the application of the saturated drug solution instead of the vesicles), when a saturated drug solution was used instead of the transfersomes suspensions, the entire drug remained bound to the gel. This confirmed that there would be no free drug present after recovering the vesicles. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles by using 50% propanol, by filtering it, and the drug amount there was determined spectrophotometrically.

Degree of Deformability

Degree of deformability is an important and unique parameter of transfersomal formulations because it differentiates transfersomes from other vesicular carriers like liposomes that are unable to cross the stratum corneum intact. The deformability study was done for the transfersomal formulation against the standard liposome preparations by using a home-built device as described by Jain et al.[18] and Cevc et al.[19] In this study, the flux of vesicles suspensions through a large number of pores of known size (through a sandwich of different polycarbonate filters with pore diameter between 200 and 50 nm, depending on the starting transfersomes suspension) was driven by an external pressure of 2.5 bar. The amount of vesicle suspension, which was extruded during 5 min was measured, and the vesicle size and the size distribution were monitored by DLS measurement before and after filtration. The experiment was performed in triplicate, and each sample was analyzed twice. The degree of deformability was calculated by using the following formula, as reported by Berge vanden et al.[20]:

$$D = J^*(r_v/r_p)^2$$

where, D = deformability of vesicle membrane
J = amount of suspension, which was extruded during 5 min
r_v = size of vesicles (after passes)
r_p = pore size of the barrier

Number of Vesicles per Cubic mm

This is the most important parameter for optimizing the composition and other process variables. Transfersomal formulation (without sonication) was diluted five times with 0.9% of NaCl solution, and the number of transfersomes per cubic mm were counted by optical microscopy by using a haemocytometer. The transfersomes in 80 small squares were counted and calculated by using the following formula.[21]

$$\text{Total no. of transfersomes per cubic mm} = \frac{\text{Total number of transfersomes counted} \times \text{dilution factor} \times 4000}{\text{Total number of squares counted}}$$

In Vitro Drug Release (Skin Permeation Study)

The in vitro skin permeation of dexamethasone from transfersomal formulation was studied by using a locally fabricated diffusion cell. The effective permeation area of the diffusion cell and receptor cell volume were 1 cm^2 and 10 mL, respectively. The temperature was maintained at $37 \pm 1^{\circ}C$. The receptor compartment contained 10 mL phosphate buffer saline (PBS) (pH 6.5) and was stirred constantly by a magnetic stirrer (Expo India Ltd., Bombay, India) at 100 rpm. Albino rat skin (4 to 5 weeks old, Sprague-Dawley strain) was mounted between the donor and the receptor compartments. The transfersomal formulation with 500 μg drug (1 mL) was applied to the epidermal surface of the rat skin. Samples were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 48 hr and analyzed by high-performance liquid chromatographic (HPLC) assay. The receptor phase was immediately replenished with an equal volume of fresh diffusion buffer. The sink condition was maintained by replacing receptor solution through the receptor compartment at the rate of 2 mL/h. Triplicate experiments were conducted for each study. In vitro skin permeation studies were conducted for different formulations, and the effect of variations in composition on permeation rate were studied. An in vitro drug release study of transfersomal formulation was repeated with a cellophane membrane by using the same method as described above.

Effect of Charge on the Amount of Drug Deposited into the Skin

To study the effect of charge on drug retention, a charge-imparting constituent was included in the selected transfersomal formulation. Dicetyl phosphate and Stearyl amine were added to the formulations for imparting negative and positive charges, respectively, as reported by Yu and Liao,[22] and the formulations were prepared by the conventional rotary evaporation sonication method as described earlier. The zeta potentials of different charged transfersomal formulations were determined by using a Zetasizer (Malvern Instrument Ltd., Malvern, U.K.).

For determining the amount of drug deposited into the skin, the method described by El. Maghraby et al.[23] was used. In this method the in vitro drug release study was performed in two stages by using the same locally fabricated diffusion cell at $37 \pm 1^{\circ}C$, as described earlier. In the first stage, PBS, (pH 6.5) 10 mL, was used as the receptor medium and a transfersomal formulation with 500 μg of drug was applied to the epidermal surface of the rat skin. Samples were withdrawn through the sampling port of the diffusion cell at predetermined intervals over 10 hr and analyzed by HPLC assay. The receptor phase was immediately replenished with an equal volume of fresh diffusion buffer. At the end of 10 hr, the donor compartment was washed five times with warm receptor fluid ($45^{\circ}C$). The second stage used 50% v/v ethanol as the receptor solution for a further period of 12 hr and was performed without any donor phase. During this stage an ethanolic receptor will diffuse into the skin disrupting the vesicular structure of any transfersomes that may have penetrated and deposited in the tissue, and, thus, releasing both transfersomes bound and free dexamethasone for collection by the receptor fluid. Use of 50% ethanol as a receptor fluid can slightly reduce the barrier nature of the stratum corneum, hence, the second stage was performed after removal of the donor to avoid any excess permeation due to penetration enhancing activity of ethanol.

In Vivo Study

The anti-inflammatory activity of dexamethasone was studied by a carrageenan-induced rat paw edema volume model as reported by Schrier et al.,[24] using plethysmograph. Male Albino rats (Sprague-Dawley strain), weighing 100–150 g, were divided into six groups of three each. After weighing the

rats individually, they were marked on the hind paw just beyond the tibiotorsal junction, so that every time the paw was dipped in the mercury column up to fixed mark to ensure constant paw volume. Initial paw volume of the rats was measured by dipping the rat paw into the mercury column, and the increase in volume due to mercury displacement was noted from the scale attached to the plethysmograph. Selected formulations (TF-DC_3, TF-SP_3, and TF-TW_3), Table 1, which showed better in vitro release profiles, were studied for their in vivo performance. The first groups of rats (control) received the topical application of normal saline solution without drug. To the second, the third, and the fourth groups of rats, transfersomal formulations containing equivalent amount of dexamethasone (100 μg) were applied on the hind paw region, whereas, the last two groups of rats received ointment and liposome containing the same amount of drug. The ointment formulation was the topical ointment with a water-washable base having the formula:

Polyethylene glycol 400	30 mg
Polyethylene glycol 4000	20 mg
Dexamethasone	1 mg

After 1 hr of topical application, 0.1 mL of 1% (w/v) carrageenan suspension in distilled water was injected in the subplantar region of the right hind paw of the rats. Paw volume was measured at 1 hr interval after carrageenan challenge by using a mercury plethysmograph by the mercury displacement method, as described above. The percentage inhibition of carrageenan-induced paw edema was calculated for each formulation by using the following equation:

$$\%\ \text{Inhibition of edema} = \frac{V_{(\text{control})} - V_{(\text{treated})}}{V_{(\text{control})}} \times 100$$

where $V_{(\text{control})}$ = mean edema volume of rats in the control group; $V_{(\text{treated})}$ = mean edema volume of each rat in the test groups.

Fluorescence Microscopy

Fluorescence microscopy was performed to confirm the penetration ability of transfersomes in comparison with liposomes. Preparing the transfersomes, as mentioned earlier, in the presence of fluorescence marker 6-carboxyfluorescein and rhodamine-123 provided the fluorescence labeling. The fluorescence marker loaded formulation was applied topically to the albino rats. After 3 hr of application, the rats were sacrificed, the skin was removed, cut into small pieces, fixed by the conventional procedure, and examined under a fluorescence microscope (Leica, DMRBE Germany). Skin not treated with any formulation served as a control.

Stability Studies

Transfersomes stability was determined at 4°C and 37°C by TEM visualization and DLS size measurement at different time intervals (30, 45, and 60 days), following vesicles preparation.

RESULTS AND DISCUSSION

The conventional rotary evaporation sonication method reported by Cevc et al.[12] and El. Maghraby et al.[13] was used to prepare the transfersomal formulation. Formulations were prepared by using different types and concentrations of surfactant. The biosurfactant sodium deoxycholate was used because of its biocompatibility; Tween-80 and Span-80 were selected because they are pharmaceutically acceptable (Table 1). Electron microscopy was used for the initial characterization of the transfersomes, where samples were negatively stained by using PTA. Transfersomes appeared as multilamellar vesicles, with the lamellae of vesicles evenly spaced to the core (Fig. 1). The size of the transfersomes was measured for formulations extruded through polycarbonate membranes, and the results are expressed as the average vesicle size for vesicles containing sodium deoxycholate, Span-80, and Tween-80, respectively (Table 1). There were insignificant differences in size between the transfersomal formulation containing different surfactants. These results correlate well for the reason that a similar method of preparation was involved where the vesicles were homogenized by extrusion through a series of 200 and 450 nm polycarbonate membranes. However, a reduction of vesicle size was observed when surfactant concentration increased above 15% w/w. This is due to the formation of a micellar structure instead of the vesicles, which are relatively smaller in size.

Entrapment efficiency is the percentage fraction of the total drug incorporated into the transfersomes. The maximum entrapment efficiency obtained was 95% for transfersomal formulation TF-SP_1. The effect of surfactant concentration in the lipid components of vesicles on the entrapment efficiency of the lipophilic model drug, dexamethasone (Fig. 2),

Table 1. Composition and characterization of transfersomal formulations.

S.No.	Formulation code	Composition PC: S (%w/w)	Characteristic	No. of vesicles per $mm^3 \times 1000$	Entrapment efficiency (%)
1.a	TF-DC_1	95: 5	+++	49	93.2 ± 0.37
1.b	TF-DC_2	90: 10	+++	46	85.3 ± 1.32
1.c	TF-DC_3	85: 15	++++	44	81.4 ± 0.84
1.d	TF-DC_4	80: 20	++	32	72.1 ± 2.34
1.e	TF-DC_5	75: 25	+	19	65.3 ± 1,34
2.a	TF-TW_1	95: 5	+++	46	89.1 ± 1.84
2.b	TF-TW_2	90: 10	+++	41	85.1 ± 0.83
2.c	TF-TW_3	85: 15	++++	39	84.3 ± 1.32
2.d	TF-TW_4	80: 20	++	26	74.1 ± 0.94
2.e	TF-TW_5	75: 25	+	17	70.3 ± 2.1
3.a	TF-SP_1	95: 5	+++	51	95 ± 1.39
3.b	TF-SP_2	90: 10	+++	50	91.5 ± 0.37
3.c	TF-SP_3	85: 15	++++	48	88.5 ± 0.86
3.d	TF-SP_4	80: 20	++	36	86.2 ± 0.1.64
3.e	TF-SP_5	75: 25	+	21	81.2 ± 0.93

Values represented as mean ± SE ($n = 3$).
+: Clear to colloidal; ++: Less turbid and colloidal.
+++: Turbid and colloidal; ++++ : Dense and colloidal.
TF-DC: Transfersomes with sodium deoxycholate.
TF-TW: Transfersomes with Tween-80.
TF-SP: Transfersomes with Span-80.

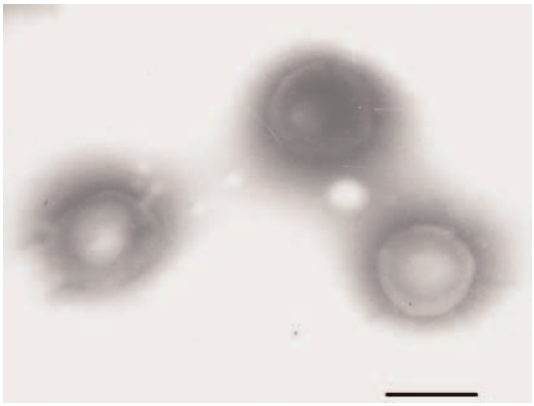

Figure 1. Visualization of transfersomal vesicles by transmission electron microscopy (magnification × 18,0000) composed of Soya PC, Span-80, and water.

clearly shows that entrapment efficiency decreased with an increase in concentration of surfactant. This is due to the possible coexistence of mixed micelles and vesicles at higher concentrations of surfactant, with the consequence of lower drug entrapment in mixed micelles.[25]

The reduction in entrapment efficiency also depended on the surfactant type. Span-80 produced a lesser effect compared with Tween-80 (Table 1). However, the pronounced effect exerted by sodium deoxycholate could be attributed to the fact that the cholate has a steroidal structure similar to dexamethasone and, thus, displaces a part of the latter from the bilayared vesicles. This displacement involves competition between species and, thus, becomes noticeable at high concentrations of sodium deoxycholate.[15]

The crucial feature of all transfersomal drug formulations, in comparison with the standard liposomes and other types of the drug-laden lipid suspensions, is the flexibility of the transfersomes. The extremely high flexibility of the membrane permits transfersomes to squeeze themselves even through pores much smaller than their own diameters (Table 2). This is due to the high flexibility of the transfersomes membrane and is achieved by judiciously combining at least two lipophilic/amphiphilic components (phospholipid plus biosurfactant), with sufficiently different packing characteristics, into a single bilayer. The resulting high-aggregate deformability permits transfersomes to penetrate the skin spontaneously and minimizes the risk of complete vesicle rupture in the skin. The negligible difference in size, after passing through a sandwich of polycarbonate membranes, indicates that these vesicles could deform or change their shape. Therefore, the rupture

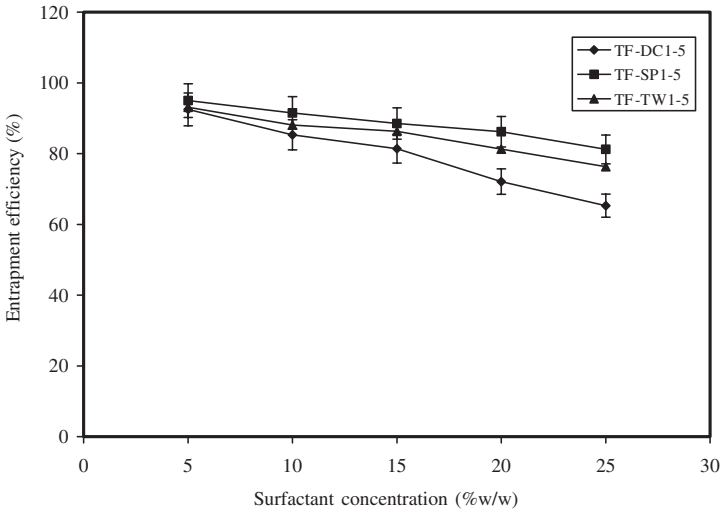

Figure 2. Effect of surfactant concentration in the lipid component of transfersomes on the entrapment efficiency of lipophilic model drug dexamethasone. Mean ± S.E ($n = 3$).

Table 2. Steady-state transdermal flux, correlation coefficient, degree of deformability of different transfersomal formulations.

Code	Transdermal flux (μg/cm^2/hr)	Correlation coefficient	Particle size (nm) (before)	Particle size (nm) (after)	Extruded vol. (mL)	Degree of deformability
TF-DC$_1$	4.41 ± 0.34	0.9826	149 ± 1.3	112 ± 1.2	4.1 ± 0.2	20.57
TF-DC$_2$	5.55 ± 0.26	0.9785	117 ± 1.5	101 ± 1.3	5.8 ± 0.3	23.66
TF-DC$_3$	7.48 ± 0.21	0.9861	119 ± 1.3	111 ± 1.1	7.3 ± 0.35	35.97
TF-DC$_4$	4.35 ± 0.32	0.9909	109 ± 2.1	92 ± 3.2	3.3 ± 0.22	11.17
TF-DC$_5$	3.05 ± 0.11	0.9877	99 ± 2.4	83 ± 3.1	2.6 ± 0.18	7.16
TF-TW$_1$	4.03 ± 0.17	0.9826	143 ± 2.8	105 ± 2.2	4.1 ± 0.22	18.08
TF-TW$_2$	4.8 ± 0.24	0.9785	149 ± 1.7	109 ± 1.5	4.3 ± 0.18	20.43
TF-TW$_3$	6.34 ± 0.19	0.9785	138 ± 1.9	121 ± 1.7	5.4 ± 0.15	31.62
TF-TW$_4$	3.62 ± 0.17	0.9909	98 ± 2.4	86 ± 3.0	3.0 ± 0.2	8.87
TF-TW$_5$	3.26 ± 0.32	0.9877	92 ± 2.6	77 ± 3.2	2.2 ± 0.4	5.21
TF-SP$_1$	5.11 ± 0.14	0.9826	138 ± 2.1	115 ± 2.0	5.0 ± 0.45	26.45
TF-SP$_2$	5.21 ± 0.26	0.9785	143 ± 1.4	126 ± 2.4	6.2 ± 0.35	39.37
TF-SP$_3$	7.81 ± 0.42	0.9861	132 ± 1.6	127 ± 1.8	8.6 ± 0.5	55.48
TF-SP$_4$	4.39 ± 0.15	0.9909	110 ± 2.3	91 ± 3.0	4.0 ± 0.3	13.24
TF-SP$_5$	4.12 ± 0.32	0.9877	92 ± 1.8	78 ± 2.4	3.6 ± 0.25	8.76
Liposome	3.36 ± 0.36	0.8321	95 ± 2.8	71 ± 2.5	1.0 ± 0.15	1.96

Values represented as mean ± SE ($n = 3$).

of these vesicles during passage is minimum as compared with control liposome preparation. These results are in agreement with the reports published in the literature.[7,20,26,27]

The transfersomal systems were studied for drug permeation across rat skin and cellophane membrane. Two different types of membrane (natural and synthetic) were taken in order to observe the effect of the nature of the membrane on permeation behavior. The studies indicated that the transfersomes bearing dexamethasone followed a mixed order release rate pattern. Initially, the permeation rate was higher (up to 6 hr), due to the release of the surface-absorbed drug, followed by nearly zero order release (up to 48 hr), and the lag time associated was more in the case of the rat skin as compared with the cellophane membrane (Fig. 3). This is possibly due to better reservoir properties of the dermis region of rat skin;

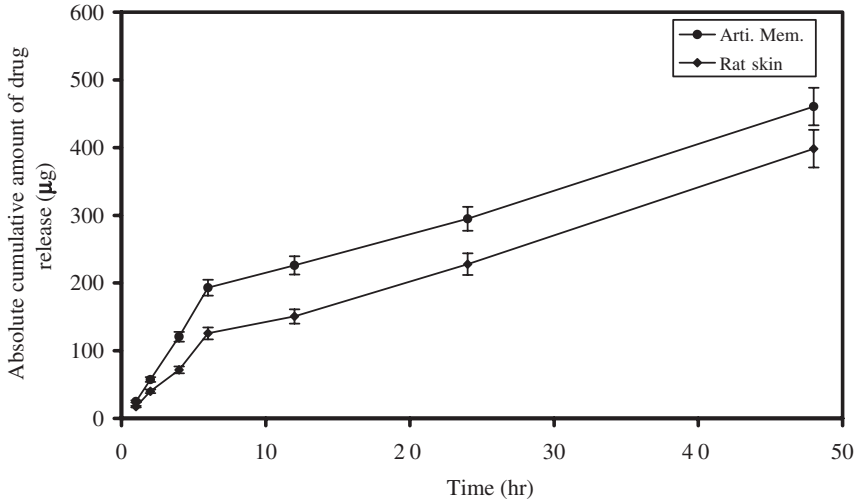

Figure 3. Effect of type of barrier, artificial vs. natural on permeability of drug from transfersomal formulation (formulation code TF-SP_3). Mean ± S.E ($n = 3$).

where-upon, transfersomes could behave as a depot, resulting in a delayed release. The skin deposition study supported this fact. The value of a dose deposited is nearly 10 times higher in the case of transfersomal formulation (59.9 ± 1.3%) than PEG ointment (6.3 ± 0.45%), which could be attributed to the difference in the mechanism of drug transport across the skin for the vesicles and the conventional formulation of a dexamethasone. This difference is due to the fact that, in contrast to a dexamethasone molecule, transfersomes are too large to enter into the cutaneous blood circulation directly; locally, they bypass the cutaneous capillary bed and get to the subcutaneous capillary bed and to the subcutaneous tissue, and here they act as a depot and sustain the drug release. The drug from transfersomes is consequently cleared less efficiently by the intradermal capillary plexus, allowing more of the drug to reach the deep subcutaneous tissue. In a conventional drug formulation (ointment), free drug movement occurs, allowing drug absorption by intradermal capillary plexus once it reaches into the dermis region of the skin and then into the systemic circulation.[28] However, in the case of cellophane membrane, no such barrier was present, and the lag time was relatively less and more of the total drug was delivered in 48 hr across the cellophane membrane (Fig. 3).

Transfersomes, when applied under suitable conditions can transfer 4 µg to 8 µg of a drug/hr/cm^2 area across the intact skin (Table 2). This value is substantially higher than that which typically is driven by the transdermal concentration gradients and other previous approaches for transdermal delivery of corticosteroids.[29,30] The probable reason for this high flux rate is a naturally occurring transdermal osmotic gradient, i.e., another much more prominent gradient is available across the skin.[5,26] This osmotic gradient is developed due to the skin penetration barrier, which prevents water loss through the skin and maintains a water activity difference in the viable part of the epidermis (75% water content) and nearly completely dry stratum corneum, near to the skin surface (15% water content).[31] This gradient is very stable because ambient air is a perfect sink for the water molecule even when the transdermal water loss is unphysiologically high. All polar lipids attract some water due to the energetically favorable interaction between the hydrophilic lipid residues and their proximal water. Most lipid bilayers thus spontaneously resist an induced dehydration.[32] Consequently, all lipid vesicles made from the polar lipid vesicles move from the rather dry location to the sites with a sufficiently high water concentration. So when a lipid suspension (transfersomes) is placed on the skin surface, which is partly dehydrated by the water evaporation loss, the lipid vesicles feel this osmotic gradient and try to escape complete drying by moving along this gradient. They can only achieve this if they are sufficiently deformable to pass through the narrow pores in the skin, because transfersomes are composed of surfactant and have better rheologic and hydration properties, which are responsible for their superior skin penetration ability. Less deformable vesicles, including standard liposomes are confined to the skin surface where they dehydrate completely and fuse, so they have less penetration power than transfersomes. Transfersomes are optimized in this respect and thus attain maximum

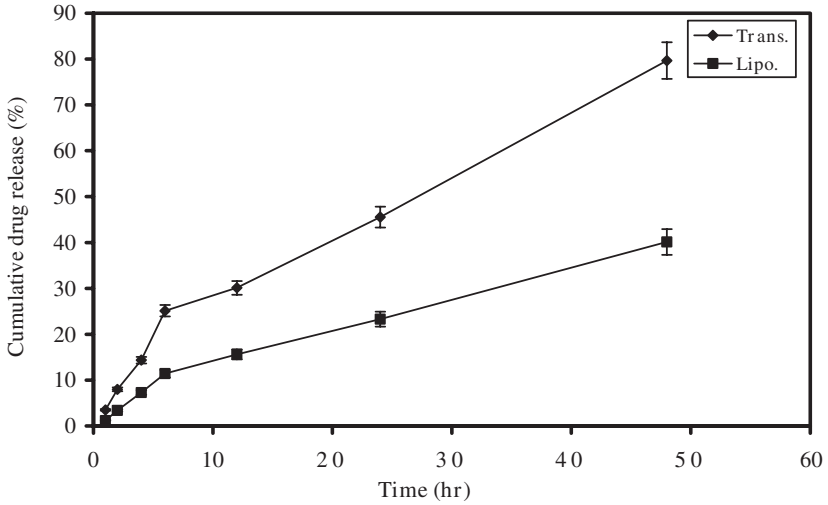

Figure 4. In vitro permeation of dexamethasone from transfersomes vs. liposomes through skin (formulation code TF-SP_3). Mean ± S.E ($n = 3$).

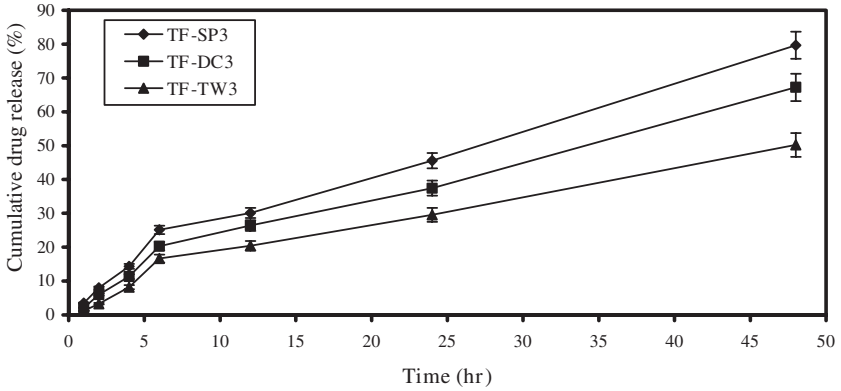

Figure 5. Effect of type of surfactant on percent cumulative drug release of lipophilic model drug dexamethasone (optimization of type of surfactant for refinement of transfersomal formulation). Mean ± S.E ($n = 3$).

flexibility and, hence, can take full advantages of the transepidermal osmotic gradient (water concentration gradient).

Figure 4 compares the in vitro drug release profile of transfersomal (TF-SP_3) and liposomal formulation, and shows that transfersomes formulations have two-to three-fold better skin permeation than the liposomal formulation. The reason for this better performance is the deformability and the ability to retain vesicle integrity, while the aggregates undergo a dramatic change in shape in comparison with liposomes, and all these characteristics allow the transfersomes to pass through the skin that is much smaller in diameter than their own diameter.[6,19,32]

Figure 5 shows the effect of the type of surfactant on percent cumulative drug release. To explain the difference in the extent of the interaction of different surfactants with the lipid bilayers, the molar ratio of the individual surfactant in vesicles may be considered. These were 1:3.6, 1:3.1, and 1:9.2 (surfactant: lipid) for sodium deoxycholate, Span-80, and Tween-80, respectively. Span 80 used at the highest molar ratio, produced a greater effect, but the differences between effects produced by sodium deoxycholate and Tween-80 were limited in spite of a considerable difference in their molar ratios. Also, comparison between Span-80 and sodium deoxycholate or Tween-80 revealed that the molar ratio is not the only factor operating. Accordingly, the HLB (hydrophilic/lipophilic balance), which gives a measure of the physicochemical properties of surfactants in terms of their affinity for, or solubility in, water or

lipids, also was considered. The HLB values of Span-80 and Tween-80 are 4.3 and 15, respectively.[33] We calculated the HLB for sodium deoxycholate to be 16.7. Based on these HLB values, the affinity for lipids is expected to be in the order of Span-80 > Tween-80 > sodium deoxycholate. Thus, at similar molar ratios, the extent of surfactant interaction with lipid bilayers should be in the following order: Span-80 > sodium deoxycholate > Tween-80, so that Span 80 should produce better effect in comparison with the other two surfactants by providing greater flexibility to the vesicle membrane. This correlates well with the results of the in vitro drug release study and the degree of deformability study (Tables 1 and 2).

With respect to drug delivery from the vesicles (Table 2), transdermal flux first increased with increasing surfactant concentration and then decreased, a common phenomenon seen with all three surfactants. These results suggested that a too low or a too high concentration of edge activators (surfactants) is not beneficial in vesicular delivery through skin and also indicated that the possible penetration enhancing effect of surfactants is not mainly responsible for improved dexamethasone skin delivery from deformable vesicles. These findings are in agreement with published data.[8,12] A possible explanation for lower drug delivery at a high surfactant concentration may be that the surfactant at high concentration decreased the entrapment efficiency and disrupted the lipid membrane so that it becomes more leaky to the entrapped drug. This will, in turn, reduce the delivery, especially if we consider the possible carrier function of these transfersomes. Another possible explanation for the obtained lower transdermal flux at a higher surfactant concentration in transfersomal formulation, particularly in case of sodium deoxycholate containing vesicles, is that at a higher concentration of surfactant, vesicles with mixed micelles coexist [surfactant con. 20% w/w of phosphatidyl choline (PC)] with only mixed micelles at (>20% w/w of PC). At a higher surfactant concentration, the transfersomal formulation was less turbid (Table 1), suggesting a similar loss of vesicular structure and formation of mixed micelles. These mixed micelle are reported to be less effective in a transdermal drug delivery as compared with a transfersomal system because micelles are much less sensitive to a water activity gradient then transfersomes. This hypothesis is supported by the report of Cevc et al.[6] who compared the penetration ability of transfersomes, liposomes, and mixed micelles by confocal laser scanning microscopy (CLSM) and

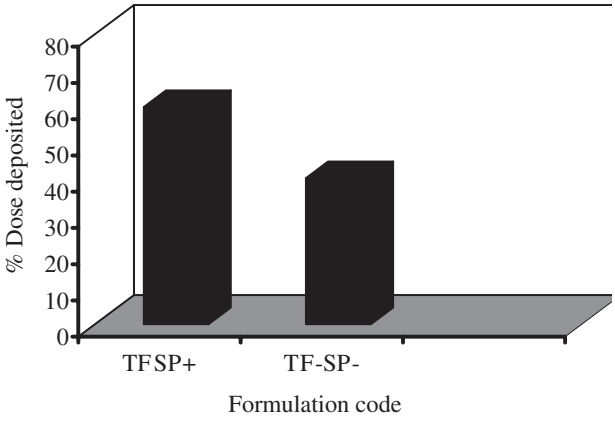

Figure 6. Effect of charge on retention of drug into skin from transfersomal formulation (formulation code TF-SP_3). Mean ± S.E ($n = 3$).

observed that mixed micelles were restricted to the top-most part of the stratum corneum and transfersomes penetrate to a deeper skin layer.

Figure 6 shows the effect of charge on the permeation of a drug across the rat skin. Comparing different surface charges in optimized transfersomal formulations (TF-SP_3), the differences in amount penetrated and permeability of dexamethasone were not statistically significant, but the skin retention of dexamethasone differed significantly. The positively charged formulation (ζ 22.8 ± 0.9) showed higher, whereas, negatively charged formulation ($\zeta - 29.6 \pm 1.2$) showed lower skin retention. This is in agreement with a report by Yu and Liao[22] who compared the drug retention of triamcinolone-loaded, different charge, liposomal formulations and found a significantly higher intradermal retention of triamcinolone than did other charge formulations because, at physiological pH, the cell surface bears a net negative charge. The method used for determining the amount of drug deposited into the skin (as described in the experimental section), enabled the calculation of permeation parameters from the first stage. It also gave a good measure of skin deposition as determined by using 50% ethanolic receptor, which will diffuse through the skin, disrupting any transfersomes structures and extracting the deposited drug from the skin. The second step was performed after removing the donor phase for two reasons; firstly, to avoid the effect of ethanol back diffusion to the surface and, thus, possible extraction of drug from the donar compartment; and secondly, to ensure that the possible effects of 50% ethanol on membrane permeability would not alter the results. This design gave a more

Table 3. Evaluation of biological activity of transfersomes, liposomes, and ointment formulation by carrageenan-induced rat paw edema model.

		% Inhibition in paw edema volume				
Group	Formulation code	1 h	2 h	3 h	4 h	5 h
Second	TF-DC_3	25.4 ± 1.2	48.1 ± 1.3	56.3 ± 2.1	62.3 ± 1.7	76.4 ± 2.2
Third	TF-SP_3	33.4 ± 2.4	55.3 ± 2.3	68 ± 1.8	78 ± 1.6	82.3 ± 2.5
Fourth	TF-TW_3	22.3 ± 1,6	33.4 ± 1.6	48.1 ± 1.9	52.3 ± 1.9	63.3 ± 2.2
Fifth	Liposome	8.16 ± 1.3	15.1 ± 1.6	22.1 ± 3.2	24.2 ± 2.1	38.3 ± 1.7
Six	Ointment	5.1 ± 1.7	12.1 ± 1.1	17.1 ± 0.9	20.13 ± 15	23.35 ± 1.7

Values represented as mean ± SE ($n = 3$).

accurate comparative measure for the deposition of the drug into a deeper layer of skin, compared with the determination of the drug in the skin by using a washing protocol. Thus, the problem of a possible failure of the washing procedure to remove transfersomes adhering to the stratum corneum surface was avoided. The above-mentioned design was reported by the El. Magharaby et al.[13,15,23] for the determination of skin deposition of estradiol and 5-FU encapsulated in transfersomes.

The in vivo performance of selected transfersomal formulations was studied by carrageenan-induced rat paw edema model. Table 3 shows that the transfersomal formulation TF-SP_3, having the surfactant concentration 15% w/w, could provide the maximum of 82.32% inhibition of paw edema, whereas, the liposomal formulation and PEG ointment prevented about 38.32% and 25.35% of paw edema, respectively. Transfersomal formulations have shown three times better percentage inhibition of paw edema than the liposomal formulation and ointment; this establishes the superior skin penetration potential of transfersomes.

Edema suppression tests have confirmed that standard liposomes deliver dexamethasone into the skin, however, the quantity is three times less than transfersomes and nearly similar to the ointment. The biological activity of liposomes probably results from the free drug permeation from the lipid vesicles into the stratum corneum. Data also demonstrate that the biological action of the transfersomal formulation is detectable nearly 60 min after the drug administration and increases with the time of action (Table 3). Similar but less clear-cut observations are made with the liposomal formulations. The optimized and highly deformable transfersomes among all tested medications ensure the best performance and control over the delivery into the intact mammalian skin. A correlation between percent inhibition of paw edema and percent cumulative drug release through skin for formulations TF-SP_3 and TF-DC_3 was attempted, and the correlation coefficient was found to be near 0.9, after 6 hr of study. It shows a good correlation between in vitro and in vivo findings (Fig. 7).

The superior penetration potential of a developed vesicular carrier was further confirmed by fluorescence microscopy. The fluorescence marker 6-carboxyflourescein does not normally get into the deeper layer of the skin when applied as a liposomal formulation; however, this dye is transported extensively and reaches to a dermal layer when applied in the form of transfersomal formulation (Fig. 8).

The storage stability testing indicated that transfersomal formulations stored at 4°C were more stable than those stored at 37°C. Average vesicles size of transfersomal formulations was found to increase on storage, which can be attributed to the possible fusion of vesicles. This effect was least in the case of formulation stored at 4°C, which indicates fusogenicity to be a temperature-dependent process and ideal temperature being 4°C. Visualization by negative-stain TEM confirmed that the vesicular structure of transfersomes persisted after storage (not shown) (Table 4).

CONCLUSION

The results of the present study showed that deformable lipid vesicles, transfersomes, improve the transdermal delivery, prolong the release, and improve the site specificity of the lipophilic model drug, dexamethasone. The formulation optimizing study shows that specific types and concentrations of surfactant are required for providing the maximum deformability to vesicle membrane. Span-80 is more effective as compared with sodium deoxycholate and

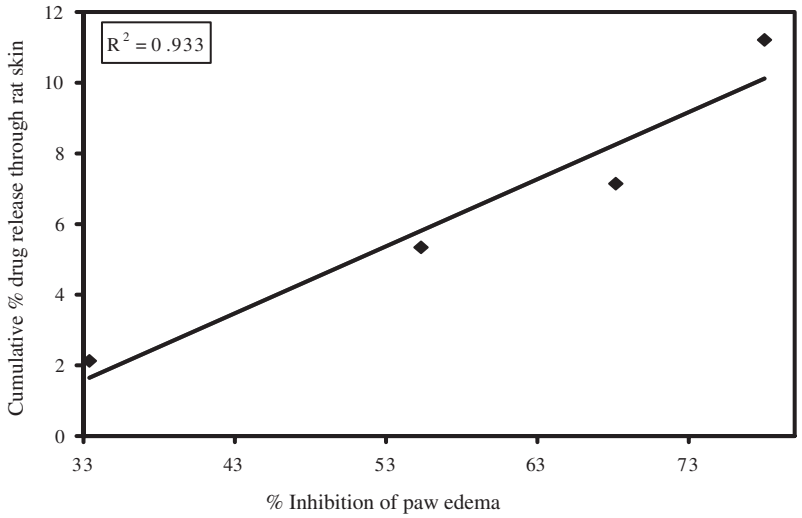

Figure 7. In vitro, in vivo correlation (formulation code TF-SP_3).

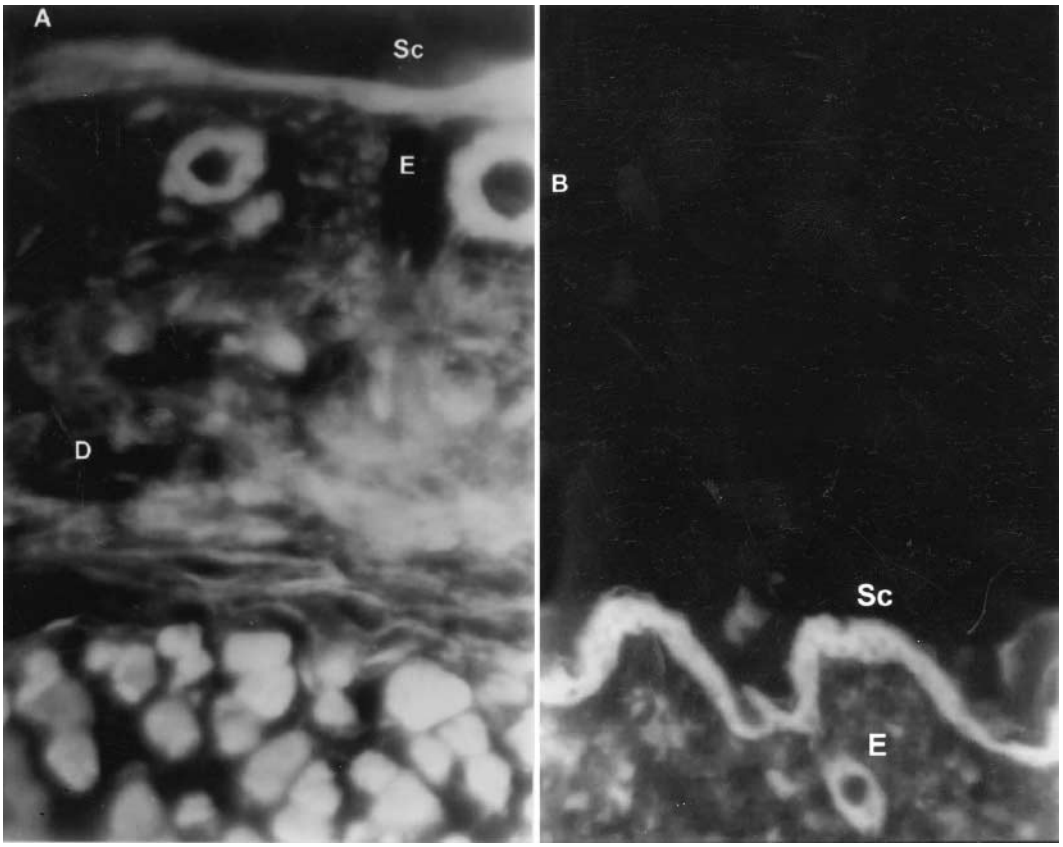

Figure 8. Penetration of 6-carboxyfluorescein as fluorescence probe from transfersomes (photomicrograph A, X450); liposomes, (photomicrograph B, X450); Sc, Stratum corneum, E, Epidermis, D, Dermis. A Shows the deposition of fluorescence probe into the dermis region when applied in the form of transfersomes and B shows the deposition of fluorescence probe into top most layer of skin stratum corneum when applied in the form of liposomal formulation.

Table 4. Stability of transfersomes vesicles.

Time (days)	Size (nm)		TEM visualization	
	4°C	37°C	4°C	37°C
1	132 ± 1.6	132 ± 1.6	Appeared	Appeared
30	139 ± 3.2	142 ± 1.9	ND	ND
45	143 ± 2.4	147 ± 1.9	ND	ND
60	145 ± 3.4	152 ± 2.7	Appeared	Appeared

Values represented as mean ± SE ($n = 3$).
(Formulation code TF-SP_3).
ND = Not determined.

Tween-80 as an edge activator and provides maximum deformability to vesicle membrane. In vivo study of optimized transfersomal formulation on suitable rodent model showed better biological anti-edema activity in comparison with liposomes and commercial ointment.

Finally, it can be concluded from the results of present study that introduction of transfersomes as a vesicular drug carrier overcomes the limitation of low penetration ability of liposomes across the skin. It creates a new opportunity for the well-controlled transdermal delivery of a number of drugs that have a problem of administration by other routes.

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