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RESEARCH PAPER

Preparation, Optimization, Characterization, and Stability Studies of Salicylic Acid Liposomes

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ABSTRACT

Salicylic acid has been used widely in the treatment of dry skin conditions and also helps reduce acne symptoms. However, it suffers from the disadvantages of being a mild to strong irritant. Hence, its control can be achieved through encapsulation in liposomes. Liposomes were prepared by the conventional thin film hydration technique as described by Bangham et al. (J. Mol. Biol., 1965). The prepared liposomal dispersions were then characterized for entrapment efficiency, particle size by transmission electron microscopy (TEM), phase transition studies by differential scanning calorimetry (DSC), and lamellarity by nuclear magnetic resonance (NMR). The results showed the formation of bilayered liposomes in the particle size range of 0.2–0.8276 μm with a maximum entrapment efficiency of 42.6%. The liposomes stored at 4–5°C demonstrated maximum stability as compared to those stored at any other temperature.

Key Words: Acne; Dry skin; Liposomes; Salicylic acid.

INTRODUCTION

Liposomes are structures consisting of one or more concentric spheres of lipid bilayers separated by aqueous buffer compartments. These spherical structures can be prepared with diameters ranging from 80 nm to 100 μm.[1]

Liposomes, a microentrapment technology, provide substantivity, improve stability, reduce side effects from actives, enhance multifunctionality, and improve ingredient compatibility.[2]

Theoretically, delivery of an active from a liposome has a predictable rate and has been shown to be much more efficiently absorbed than from the corresponding free active. It has also been established that as the liposome's ring[3] penetrates into the skin, it gradually breaks down allowing the release of active ingredients, enhancing their penetration into the skin.

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As each ring dissolves, another emerges, and the delivery of active continues for a considerable time, thus giving sustained effect.

The discovery of liposomes has opened new opportunities for novel applications in topical drug delivery. Their efficacy as vectors and their ability to ameliorate stratum corneum structure have ensured their success.

In recent years, different drugs have been incorporated into liposomes for topical delivery. Sunscreen liposomes[4] containing 22.5% ethylhexyl methoxycinnamate and free radical scavenger tocopheryl acetate are manufactured by collaborative laboratories.

Topical liposomal gel of tretinoin was prepared for the treatment of acne in 2000.[5] Vitamin A and various enzymes have also been incorporated into liposomes. Liposomes containing retinoids and vitamin C have also been tried.[6]

Salicylic acid was chosen as the model drug for this study. Over the years it has been widely used in the treatment of dry skin conditions and also helps reduce acne symptoms.[7] However, it suffers from the disadvantages of being a mild to strong irritant.[8] Hence, the control of the irritant potential can be achieved through encapsulation in liposomes. The entrapped salicylic acid is released at a controlled rate over an extended period of time, thus reducing both the irritant potential and the frequency of application of the drug. Secondly, the liposomes are known to enhance skin hydration in dry skin conditions due to the similarity between liposome components and cutaneous lipids, thus making liposomes a novel application in cosmeceuticals and pharmaceuticals.

The objective of this study was to prepare and characterize salicylic acid liposomes and carry out stability studies of the same.

MATERIALS AND METHODS

Materials

Salicylic acid (Loba Chemie) was used as the model drug. Soyabean lecithin (Hi-Media) and cholesterol (Loba Chemie) were employed as the lipids. Chloroform (E-Merck) was used as the solvent for the lipid phase. The aqueous phase was composed of phosphate buffer pH 7.4 and propylene glycol (SQ. L.R., Qualigens). All other chemicals were of analytical grade. Distilled water was used in the entire study.

Method

Conventional thin film hydration technique as described by Bangham et al.[9] was used. The rotary vacuum evaporator (Superfit, Rotovap) was used to form the lipid film as well as to hydrate the film.

Step I: Drying of the Lipid Phase

The materials constituting the lipid phase, i.e., lecithin and cholesterol, were weighed and dissolved in 10 mL chloroform. The solution was transferred to the flask of the Rotovap. The flask was attached to the rotary evaporator, evacuated and rotated at the required rpm. The process was allowed to continue until all the solvent had evaporated from the solution and a dry lipid film had deposited on the walls of the flask. The flask was rotated under vacuum for additional 30 min after the dry residue first appeared. The flask was immersed in a thermostated water bath with a temperature of 45–50°C. After stopping the rotation of the flask, it was flushed with nitrogen to remove the last traces of solvent and oxygen.

Step II: Hydration of the Lipid Film

Aqueous phase containing 50% propylene glycol was used. As salicylic acid is practically insoluble in water, 50% propylene glycol was required to maintain the drug in solution.

Propylene glycol was chosen as a cosolvent due to the following reasons :

(i) Propylene glycol is a humectant and it is known that liposomes containing propylene glycol provide better moisturizing benefits.[10]
(ii) As a humectant, it is known to compete with other components in the formulation for the available water. This helps to prevent microbial growth.[11]
(iii) It is nonvolatile.
(iv) It is less expensive than glycerine.

Specified amount of salicylic acid was dissolved in the aqueous phase with the cosolvent. This aqueous phase was then purged with nitrogen to remove traces of oxygen and then added to the flask. The flask was attached to the evaporator again and secured in position with a clip. It was rotated at the specified rpm and a temperature of 45–50°C. It was left rotating for 30 min or until all the lipid film

had been removed from the walls of the flask and had given a homogeneous dispersion. The dispersion was allowed to stand for further 2 h at room temperature to complete hydration.

Optimization

The optimization of the component concentrations and the process variables was done using a 2^3 factorial design (see Table 1).

The three factors selected were

Factor A: Lecithin concentration.

Factor B: Cholesterol concentration.

Factor C: Speed of rotation (rpm) of Rotovap.

The effect of these three factors was studied on the response, viz.

Entrapment Efficiency of Drug

Each factor was studied at two levels upper level (+) and a lower level (−). The levels selected for each parameter were as shown in Table 1b.

The concentration of salicylic acid (2%) and the solvent system ratio (i.e., concentration of phosphate buffer pH 7.4:concentration of propylene glycol) was kept constant at 1:1 throughout the experiment.

The setup of the 2^3 factorial design was as shown in Table 1a.

The eight batches of liposomes were then formulated according to the factorial design in Table 1b. Each experiment was repeated six times on six different days ($n = 6$) and all the batches were characterized for their percentage entrapment efficiency, and transmission electron microscopy (TEM) was carried out. The optimized batch was selected and the final characterization was carried out.

The experimental data, were statistically analyzed by application of Yate's treatment.[12]

Separation of the Nonentrapped Salicylic Acid

For the separation of nonentrapped salicylic acid, the liposomal dispersion was subjected to centrifugation on a laboratory centrifuge (Remi R8C) at $3333 \times g$ for 30 min. After centrifugation, the clear supernatent was siphoned off carefully to separate the nonentrapped salicylic acid leaving behind the liposomes with the entrapped salicylic acid in the sediment. The sediment was resuspended in fresh phosphate buffer pH 7.4 and the liposomal dispersion (free of the nonentrapped salicylic acid) was stored at 4°C in glass vials.

Table 1a. Optimization of liposomes: 2^3 factorial design set up.

Expt. no	Trial	Batch no	A	B	C	PC/cholesterol molar ratio
1	1	L-1	−	−	−	13.3:1
2	A	L-2	+	−	−	28.33:1
3	B	L-3	−	+	−	6.66:1
4	C	L-4	−	−	+	13.3:1
5	AB	L-5	+	+	−	14.1:1
6	AC	L-6	+	−	+	28.33:1
7	BC	L-7	−	+	+	6.66:1
8	ABC	L-8	+	+	+	14.1:1

Table 1b. Key for 2^3 factorial experimental design.

		Levels	
Factor	Parameters	Lower (−)	Higher (+)
A	Lecithin concentration	5%	10%
B	Cholesterol concentration	0.25%	0.50%
C	Speed of rotation (rpm) of Rotovap	80 rpm	120 rpm

Characterization of Liposomes

All the prepared batches (L-1 to L-8) were evaluated for the following attributes.

Entrapment Efficiency

Similar procedure used for separation of non-entrapped salicylic acid was used for determining the entrapment efficiency.

Aliquots (1 mL) of liposomal dispersion were subjected to centrifugation on a Laboratory centrifuge (Remi R 8 C) at 5000 rpm.

The clear supernatent was siphoned off carefully to separate the nonentrapped salicylic acid and the absorbance recorded at 295.2 nm. Sediment was diluted to 100 mL with methanol to lyse the liposomes and the absorbance was recorded at 295.2 nm.

Amount of salicylic acid in supernatent and sediment gave a total amount of salicylic acid in 1 mL dispersion.

Each time, a blank containing blank liposomes was treated in the same manner to account for any absorbance due to the lipid components.

The entrapment efficiency was thus calculated as follows:

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of salicylic acid in sediment}}{\text{Total amount of salicylic acid added sample}} \times 100$$

Particle Size by Transmission Electron Microscopy

The liposomal dispersions were observed under a transmission electron microscope (Zeiss, EM 109). Copper grids, double coated with Formvar, and carbon were used to examine the samples. The liposomal dispersions were diluted five times with distilled water in Eppendorf tubes with a 200-μL fixed volume pipette (Bioscience). A drop of the diluted sample was placed on the coated side of the grid and kept settling for 5 min. The grids were then blotted on a filter paper and stained with aqueous phosphotungstic acid (1%) (Ted Pella Co, Inc.) and kept for 3 min. The grids were then rinsed with distilled water to wash off the excess stain and dried at room temperature. The grids were then placed in the sample inlet chamber of the TEM and observed.

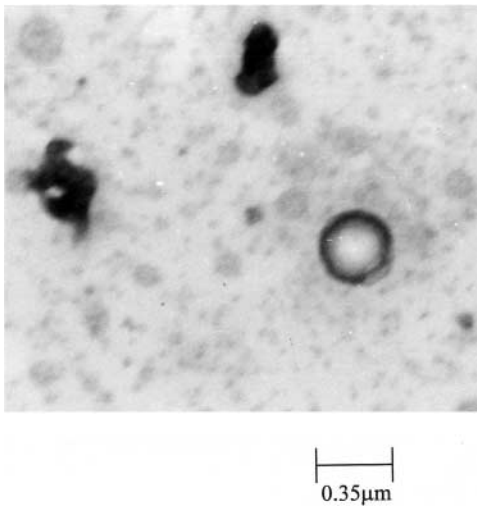

Figure 1. Transmission electron micrograph of salicylic acid liposomes (Batch: L-6, Factor: AC, Mangnification: 24,000×).

Table 2. Particle size and appearance of salicylic acid liposomes in transmission electron microscopy (TEM).

Batch no.	Particle size (μm)	Appearance
L-1	0.8276	Broken lipid film
L-2	0.3333	Uniform spherical vesicles
L-3	0.8199	Broken lipid film
L-4	0.25	Uniform spherical vesicles
L-5	0.20	Uniform spherical vesicles
L-6	0.50	Uniform spherical vesicles
L-7	0.2713	Uniform spherical vesicles
L-8	0.3333	Uniform spherical vesicles

A series of photographs were recorded avoiding overlap of the areas.

The TEM micrographs of the optimized batch (Batch L-6) are depicted in Fig. 1.

The particle size of the liposomes was calculated from the photographs using "the original slide Guide™ Ultrastructure size calculator" (Ted Pella, Inc., The Microscopy Supply Center, Redding CA, USA).

The particle size is depicted in Table 2.

Phase Transition Studies by Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) thermograms of the samples were recorded on a Du Pont 910

differential scanning calorimeter. Thermograms of both blank and salicylic acid liposomal dispersions were recorded individually (Figs. 2a–c).

The liposomal dispersions were weighed in an aluminum cuvette and sealed with an aluminum lid. The cuvette was placed in the DSC and heated from 20 to 200°C at a heating rate of 10°C/min in nitrogen atmosphere.

The scan was recorded and plotted showing heat flow (w/g) on the Y-axis and temperature (°C) on the X-axis. The plots are shown in Figs. 3(a) and 3(b).

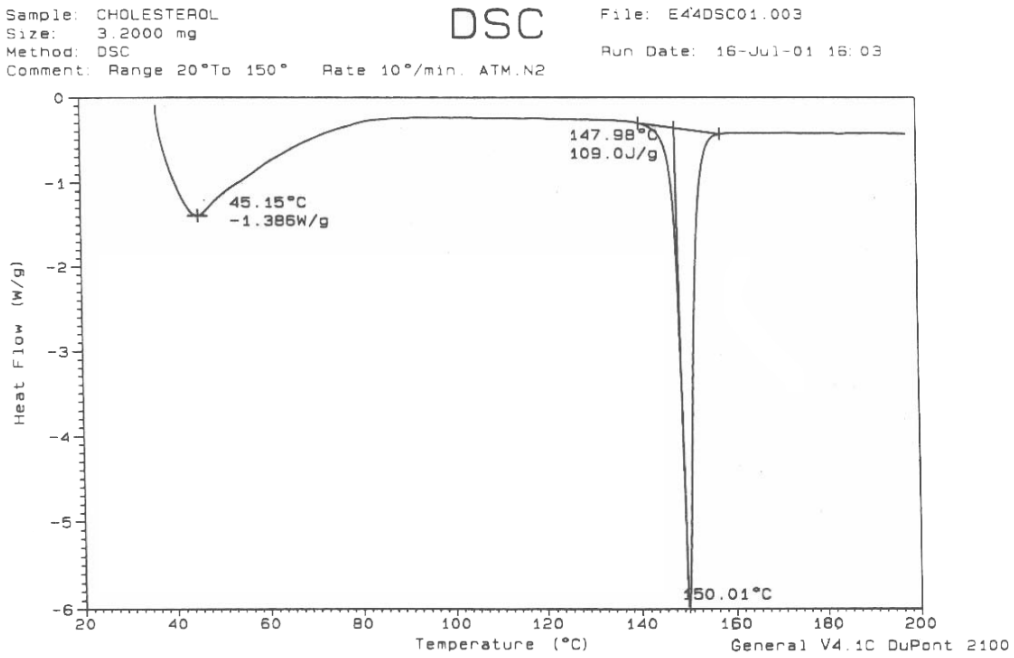

Figure 2a. DSC themogram of cholestrol.

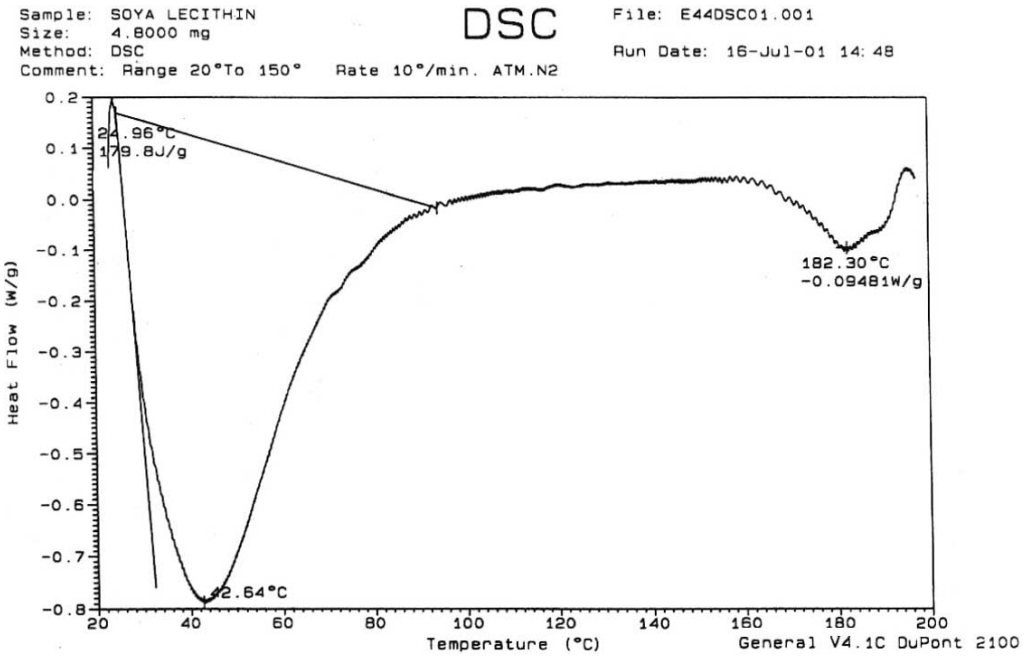

Figure 2b. DSC themogram of lecithin.

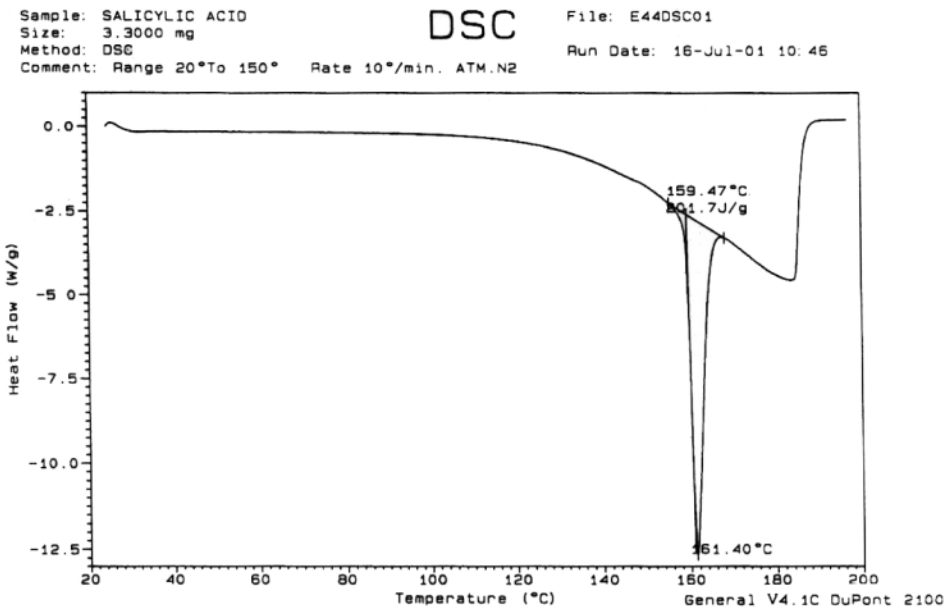

Figure 2c. DSC themogram of salicylic acid.

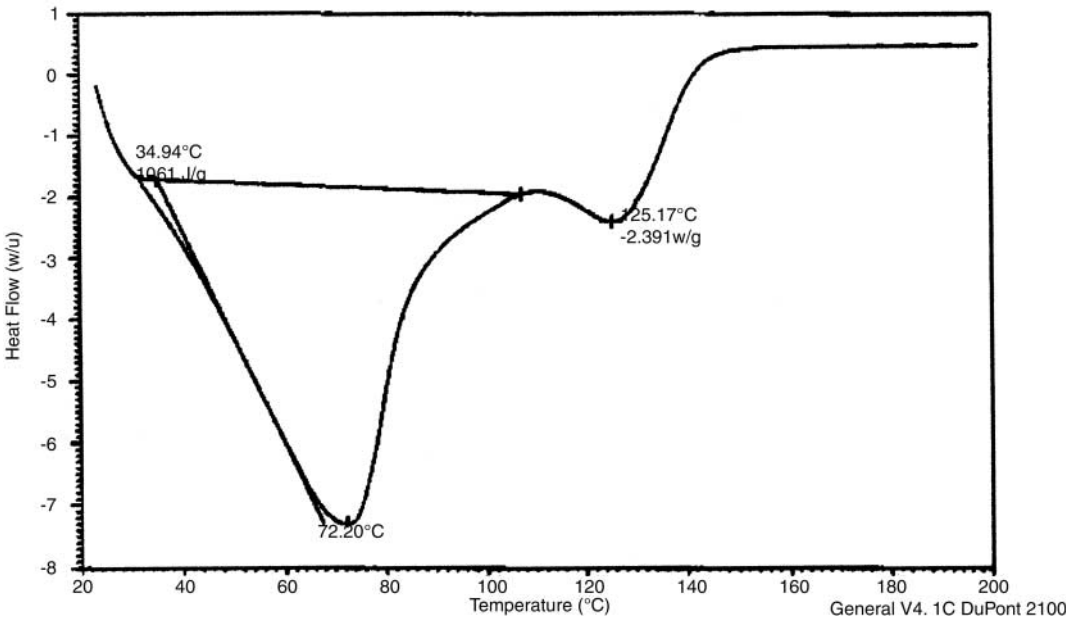

Figure 3a. DSC themogram of blank unloaded liposomes (Batch L-6).

Lamellarity by Nuclear Magnetic Resonance (^{31}P NMR)

The liposomal dispersion was subjected to ^{31}P-NMR studies using a Bruker amx-500 spectrometer.

The spectra were individually recorded before and after the addition of Mn^{2+}, which is a band broadening agent.

Sample Preparation 1

Liposomal Samples Before Addition of Mn^{2+}. Liposomal dispersion (50 μL) was withdrawn with the help of a micropipette. The volume was then made up to 100 μL with the help of deuterated water (D_2O). This was then introduced into the NMR sample tube. The sample tube was sealed and the contents were mixed. This was used to record the spectra.

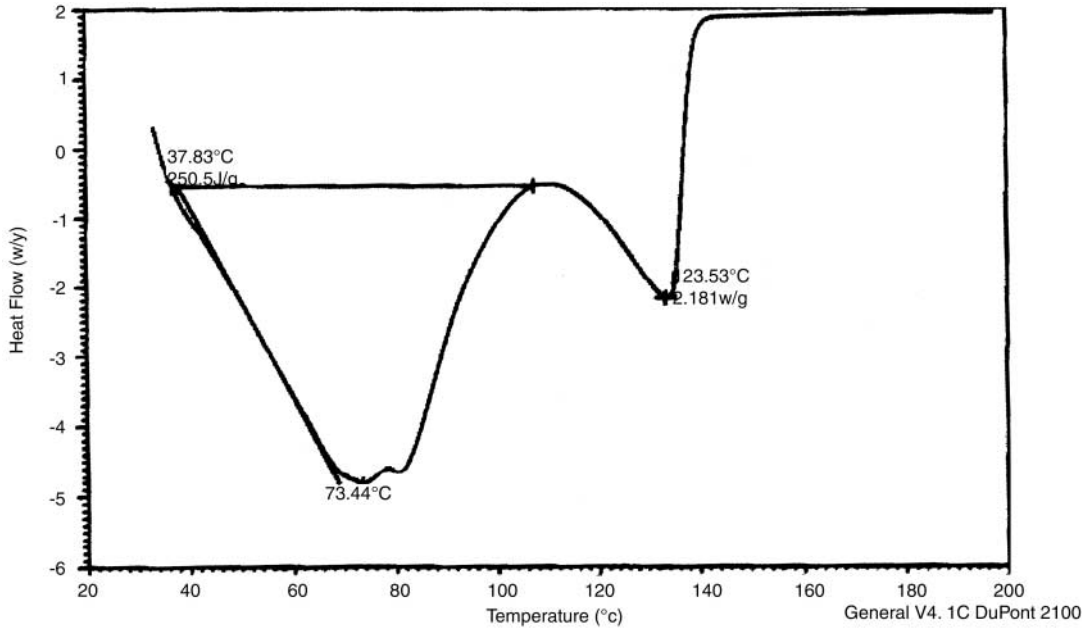

Figure 3b. DSC themogram of salicylic acid loaded liposomes (Batch L-6).

^{31}P NMR Spectra 1. The spectra were recorded using 85% H_3PO_4 (phosphoric acid) as the reference standard.

The scan has been depicted in Fig. 4(a).

Parameters used			
Probe	5 mm Multinu	Pulse program	Zgpg
Time domain (TD)	16,384	Solvent	Deuterated water (D_2O)
Number of scans (NS)	400	Spectral width in Hertz (SWH)	41,666.668 Hz
Receiver gain (RG)	1,024	Delay (D1)	3.0 sec
90° Pulse width (P1)	10 μsec	Nucleus	^{31}P

Sample Preparation 2

Liposomal Sample After Addition of Mn^{2+}. Mn^{2+} ions were added in the form of manganese chloride ($MnCl_2$) to sample preparation 1 in the NMR tube and the contents were thoroughly mixed. This was used to record the spectra.

^{31}P NMR Spectra. The spectra were recorded using 85% H_3PO_4 as the reference standard.

The scan is depicted in Fig. 4(b).

Parameters used			
Probe	5 mm Multinu	Pulse program	Zgpg
Time domain (TD)	16,384	Solvent	Deuterated water (D_2O)
Number of scans (NS)	10,240	Spectral width in Hertz (SWH)	41,666.668 Hz
Receiver gain (RG)	1,024	Delay (D1)	5.0 sec
90° Pulse width (P1)	10 μsec	Nucleus	^{31}P

Release of Salicylic Acid from Liposomes

The method developed by Zuber et al. for topical dosage forms was used for determining the in vitro release.

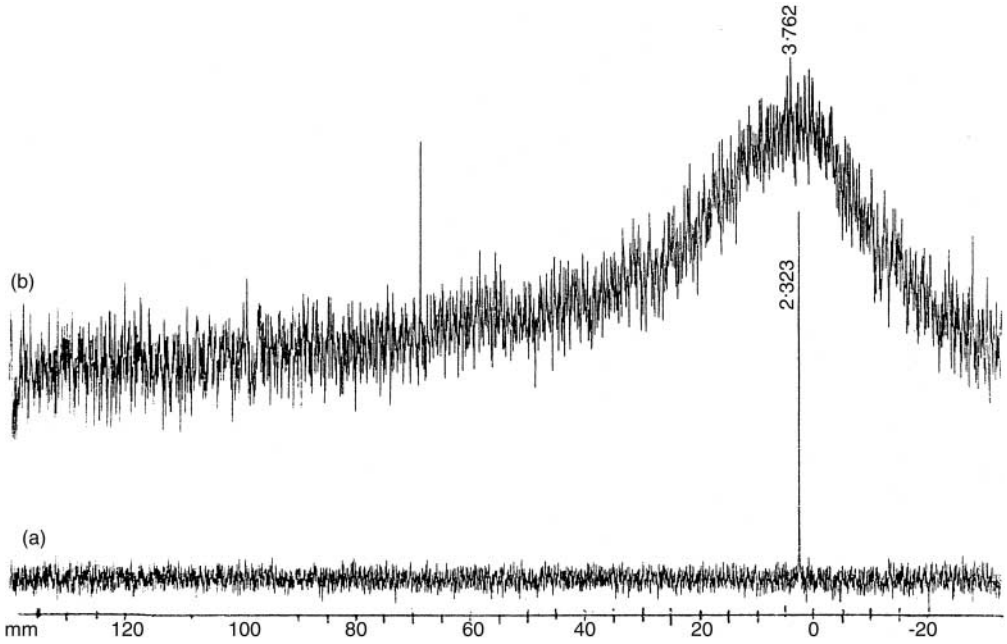

Figure 4. ^{31}P NMR of salicylic acid liposomes (Batch L-6). (a) Before addition of Mn^{2+} ions; (b) after addition of Mn^{2+} ions.

The release studies were carried out in a 500-mL beaker containing 400 mL of the medium. The medium was stirred using a magnetic needle. Dialysis membrane (Hi-Media) was utilized as a barrier to isolate the donor and the receptor phase.

Phosphate buffer pH 7.4 was utilized as the medium for determining the release of salicylic acid from liposomes.

Phosphate buffer pH 7.4 (400 mL) was placed in a 500-mL beaker. The beaker was assembled on a magnetic stirrer (Remi Equipments) and the medium was equilibrated at $37 \pm 5^{\circ}C$. Dialysis membrane (Hi-Media) measuring 5 inches was taken and one end of the membrane was sealed with the help of a close-fitting clip. The liposomal dispersion after separation of nonentrapped salicylic acid was filled in the dialysis membrane and the other end closed with another clip. The dialysis membrane containing the sample was suspended in the medium with the help of a Rhetort stand.

Aliquots of 5 mL were withdrawn at intervals of 0, 0.5, 1, 2, 3, 4, 5, and 6 h. They were filtered after withdrawal and the apparatus was immediately replenished with 5 mL of the fresh buffer medium.

The 5-mL aliquots were transferred to the cuvette of the UV-visible spectrophotometer after adjusting the blank reading with phosphate buffer pH 7.4. The absorbance of this solution was recorded at 295.2 nm and the concentration of salicylic acid at each time interval was extrapolated from the standard curve of salicylic acid in phosphate buffer pH 7.4.

The data of release of salicylic acid from liposomes and the release profiles are depicted in Table 3 and Fig. 5.

Microbiological Studies (Total Microbial Count) of the Final Formulation of Liposomes (Batch L-6)

The total microbial count (TMC) of the sample was determined by the plate count method as described in I.P. 1996.[13]

Skin Deposition Studies

The dispersion containing salicylic acid in liposomes was subjected to diffusion studies. A dispersion containing 2% w/w plain salicylic acid was also prepared and the diffusion studies carried out in the same manner for comparison.

A Franz diffusion cell with a 2.269 cm^2 diffusion area was used for the diffusion studies. The cell had an outer jacket through which water thermostated at $37^{\circ}C$ was circulated by means of silicone tubing

Table 3. Release of salicylic acid from liposomes (Batches L-1 to L-8).

Time of release (hours)	Mean cumulative % release ($n = 4 \pm$ S.D.)					
	L-2	L-4	L-5	L-6	L-7	L-8
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	13.71 (±0.4189)	16.57 (±0.4515)	16.45 (±0.377)	10.84 (±0.1154)	17.71 (±0.4929)	14.6 (±0.4704)
1	25.575 (±0.5062)	28.60 (±0.9356)	28.41 (±0.4343)	22.88 (±0.6022)	32.54 (±2.2008)	26.26 (±0.8076)
2	37.24 (±0.2470)	42.21 (±0.7257)	41.04 (±0.6478)	35.00 (±0.8577)	46.27 (±0.7932)	37.92 (±0.6559)
3	50.46 (±0.4287)	55.39 (±0.9531)	53.55 (±0.5934)	47.93 (±1.2497)	58.06 (±0.6625)	52.16 (±1.0518)
4	61.36 (±0.8446)	67.49 (±0.6013)	64.96 (±0.8874)	58.57 (±3.2906)	70.83 (±0.7677)	62.45 (±0.9656)
5	74.25 (±0.5579)	81.44 (±1.0967)	77.54 (±0.5618)	72.09 (±0.0921)	84.59 (±0.6408)	76.30 (±0.4797)
6	91.165 (±1.1045)	95.78 (±1.3178)	95.63 (±0.6053)	85.73 (±0.5520)	95.91 (±0.5223)	93.01 (±0.8938)

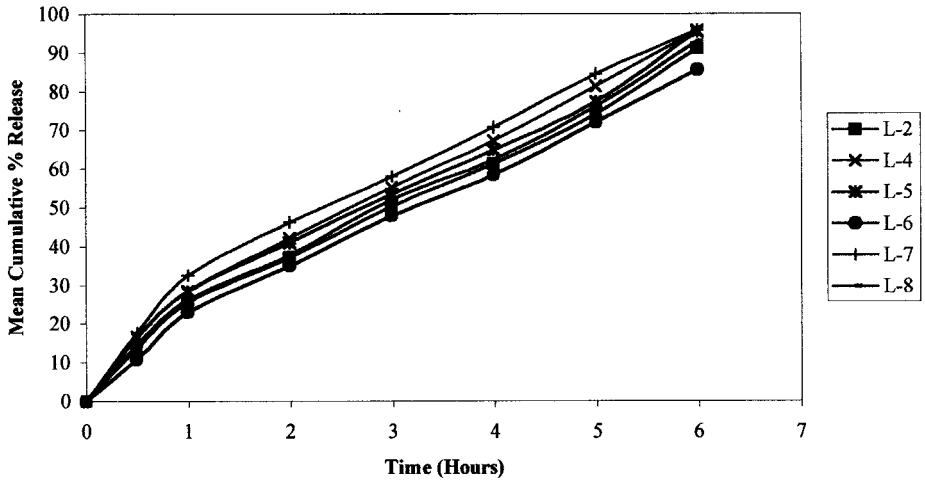

Figure 5. Release profiles of salicylic acid from liposomes (Batches L-1 to L-8).

connecting to a constant temperature water bath with the inlet and outlet ports of the jacketed glass cell. The receptor portion of the diffusion cell had an effective volume of 33 mL (as determined by a calibrated pipette) and an effective area of 2.269 cm^2 (as determined by Vernier callipers).

In the present study, porcine skin was used as the diffusion barrier. The average thickness of skin as determined by Vernier callipers was 2.0 mm. The skin sample was placed between the donor and receptor compartments with the dermis side of the skin facing the receptor solution. The two compartments were clamped together by stainless steel clamp.

The formulation (2.0 mL) was applied to the skin and spread over the 2.269 cm^2 test area with the tip of a pipette. The dermal surface of the skin was perfused with receptor phase solution (pH 5.0 phosphate buffer) and the donor surface remained unoccluded.

The diffusion cell was mounted on a magnetic stirrer (Remi) and the receptor solution was stirred using a 0.5-cm magnetic needle.

Serial sampling of the receptor compartment was performed at specified time intervals of 1, 2, 3, 4, 5, 6, 24, 48, and 72 h. At each time interval, 5 mL of the receptor phase solution was removed and analyzed by recording the absorbance at 295.2 nm on a UV-visible spectrophotometer (Jasco UVI Dec-550) after adjusting the blank reading with the buffer solution. The diffusion cell was replenished with 5 mL of the fresh medium each time after withdrawal of the aliquots. The study was continued for 72 h and the amount of salicylic acid diffused was analyzed at all sampling time points. The results are given in Table 10(a).

At the end of the study (72 h), the residual salicylic acid remaining on the surface was removed by washing the surface three times with the medium. The washings were analyzed for residual

salicylic acid. The skin was digested with 10 mL of methylene chloride overnight at 40°C and analyzed for the salicylic acid content in the skin. The results of skin retention are given in Table 10(c).

Stability Studies of Liposomes (Optimized Batch L-6)

To determine the stability of liposomes, the liposomal dispersion-optimized batch was stored in air tight sealed vials at various temperatures 4–5°C, room temperature (R.T.), 37, 45, and 55°C. The samples were kept in stability ovens (Meta-Lab scientific industries) for 37, 45, and 55°C and in a refrigerator for 4–5°C.

The liposomal dispersions were sampled at regular intervals of 0, 2, 4, 8, and 12 weeks and tested for the following attributes at each temperature: (i) signs of sedimentation or creaming if any, and (ii) change in the color of the dispersions.

The observations are recorded in Table 4.

Transmission electron microscopy was done after 12 weeks of storage at each temperature and the changes in the particle size of the samples were recorded.

The results are depicted in Fig. 6a–e and Table 5.

Extent of Leakage

To calculate the extent of leakage, the entrapment efficiency after storage of the sample was calculated and then correlated with the extent of leakage.

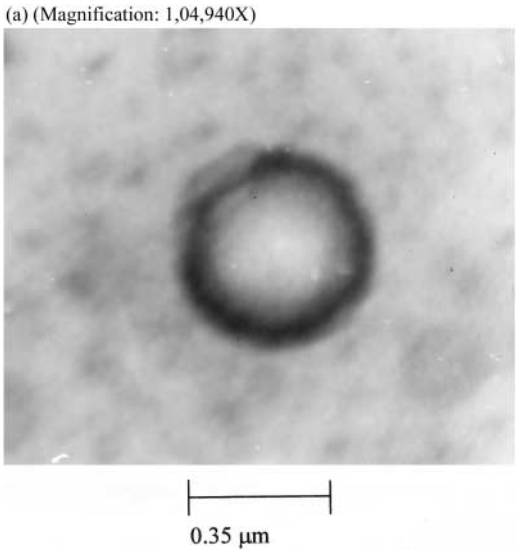

Figure 6. Stability studies—Transmission electron micrograph of salicyclic acid liposomes (Batch L-6) after 12 weeks of storage at (a) 4–5°C, (b) room temperature (R.T.), (c) 37°C, (d) 45°C, and (e) 55°C.

Table 4. Stability studies of liposomes (Batch L-6)—visual appearance and observations.

Time (weeks)	Temperature	Signs of sedimentation or creaming if any
2–4	4–5°C	No sedimentation or creaming
4		No sedimentation or creaming
8		No sedimentation or creaming
12		No sedimentation or creaming
2	Room temp.	No sedimentation or creaming
4		No sedimentation or creaming
8		No sedimentation or creaming
12		Slight sedimentation observed
2	37°C	No sedimentation or creaming
4		No sedimentation or creaming
8		Slight sedimentation observed
12		Slight sedimentation observed
2	45°C	No sedimentation or creaming
4		No sedimentation or creaming
8		Slight sedimentation observed
12		Slight sedimentation observed
2	55°C	No sedimentation or creaming
4		Slight sedimentation observed
8		Remarkable sedimentation observed
12		Remarkable sedimentation observed

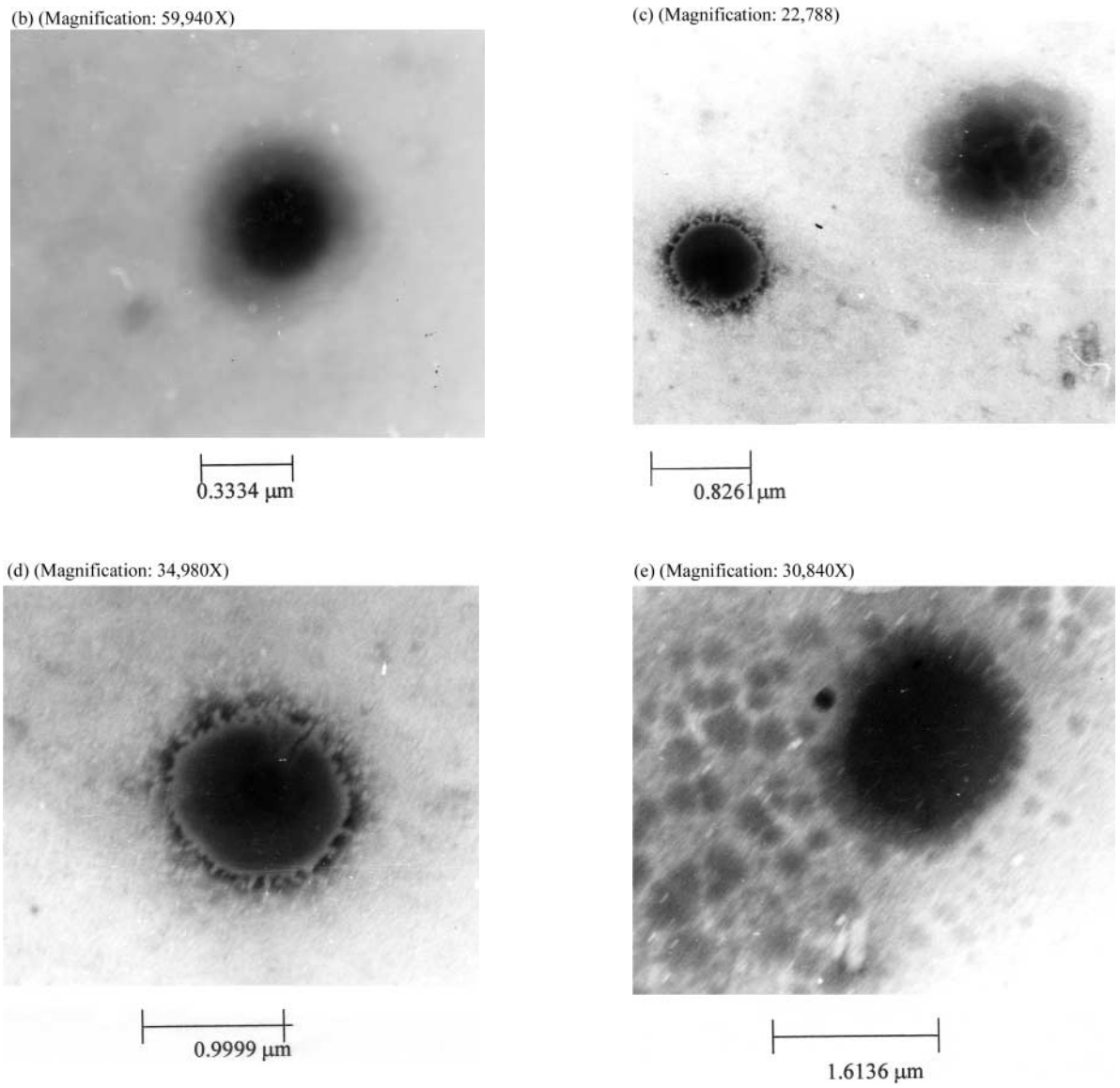

Figure 6. Continued.

Table 5. Stability studies—particle size profiles and microscopic observations by transmission electron microscopy (TEM) after 12 weeks of storage (Batch L-6).

Temperature	Particle size (μm)	Microscopic observations
4–5°C	0.35	Uniform appearance of liposomes and maintenance of liposome structure
Room temp.	0.3334	Slight leakage of the active observed around the liposome
37°C	0.8261	Outer layer of the lipsome disrupted with leakage of the active
45°C	0.9999	Greater disruption of outer layer of liposome with increased leakage of the active
55°C	1.16136	Disruption of the entire liposome structure and maximum leakage of the active seen

Table 6. Stability studies—extent of leakage of salicylic acid from liposomes (Batch L-6).

Time (weeks)	Temperature	Amount retained (%)	Amount leaked (%)
0	Room temp.	100	0
2	4–5°C	98.98	1.02
4		98.12	1.88
8		96.52	3.48
12		95.99	4.01
2	Room temp.	95.00	5.0
4		87.32	12.68
8		82.55	17.45
12		80.0	20.0
2	37°C	92.98	7.02
4		85.05	14.95
8		73.31	26.69
12		68.80	31.2
2	45°C	86.5	13.5
4		61.29	38.71
8		38.04	61.96
12		20.07	79.93
2	55°C	78.2	21.8
4		51.89	48.11
8		41.66	58.34
12		7.15	92.85

Aliquots (2 mL) of the liposomal dispersion were subjected to centrifugation on a laboratory centrifuge (Remi R 8 C) at 5000 rpm.

The clear supernatent was siphoned off carefully to separate the leaked out salicylic acid from liposomes, if any, on storage. The absorbance of the supernatent was recorded at 295.2 nm.

Methanol (2 mL) was added to the sediment to lyse the liposomes and the absorbance recorded at 295.2 nm.

Amount of salicylic acid in supernatent and sediment gave total amount of salicylic acid in 2 mL dispersion. The amount retained was then calculated as follows:

$$\%\ \text{Retained} = \frac{\text{Amount of salicylic acid in sediment}}{\text{Total amount of salicylic acid in sample}} \times 100$$

The extent of leakage of salicylic acid from liposomes is thus calculated as follows:

$$\text{Extent of leakage} = \text{Initial entrapment efficiency of sample (\%)} - \%\ \text{Retained after storage}$$

Results are depicted in Table 6 and Fig 7.

Microbiological Studies—Total Microbial Count

The TMC of the samples was determined after 12 weeks to determine the microbial load on the preparation on storage.

The procedure used was described previously.

RESULTS AND DISCUSSIONS

Entrapment Efficiency of Liposomes

Table 7(a) shows the entrapment efficiency of batches L-1 to L-6 prepared by using 2^3 factorial design. The entrapment efficiency was found to be in the range of 6.256 (±0.00516)–42.6 (0.000)% with the highest entrapment efficiency of 42.6 (0.00)% shown by batch L-6.

Liposome Particle Size by Transmission Electron Microscopy

TEM confirmed the formation of liposomes. The scanning of the grids showed the presence of spherical vesicles. The particle size range (Table 2) was between 0.2 and 0.8276 μm, which was found suitable for skin

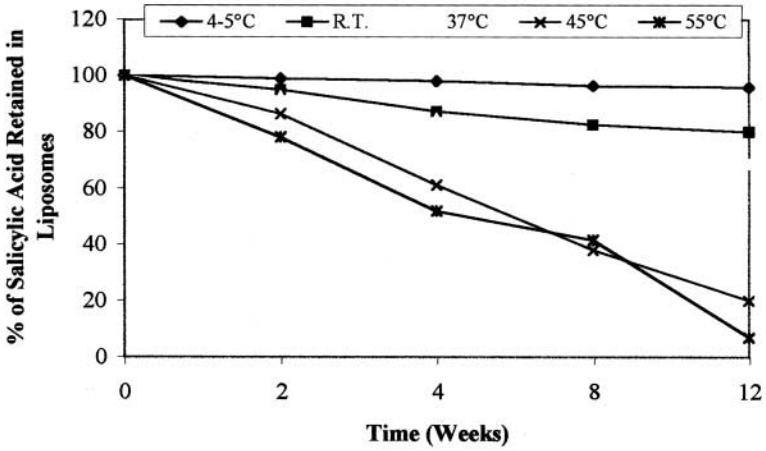

Figure 7. Percentage salicylic acid retained temperatures.

Table 7a. Values of % entrapment efficiency of salicylic acid in liposomes obtained by 2^3 factorial experimental design.

Expt. no.	Trial	Batch no.	Lecithin conc. (A)	Cholesterol concentration (B)	Speed of rotation (rpm) (C)	% Entrapment efficiency ($n=6\pm$S.D.)
1	1	L-1	−	−	−	6.256 (±0.00516)
2	A	L-2	+	−	−	39.31 (±0.5477)
3	B	L-3	−	+	−	5.205 (±0.005477)
4	C	L-4	−	−	+	31.621 (±0.00408)
5	AB	L-5	+	+	−	34.28 (0.00)
6	AC	L-6	+	−	+	42.60 (0.00)
7	BC	L-7	−	+	+	25.618 (±0.1918)
8	ABC	L-8	+	+	+	36.366 (±0.02581)

Table 7b. Key for 2^3 factorial experimental design.

		Levels	
Factor	Parameters	Lower (−)	Higher (+)
A	Lecithin concentration	5%	10%
B	Cholesterol concentration	0.25%	0.50%
C	Speed of rotation (rpm) of Rotovap	80 rpm	120 rpm

delivery, as the thickness of stratum corneum is around 10 μm, thus making it easily possible for the penetration of liposomes. TEM of Batch L-1 did not show the formation of liposomes. Batch L-3, having low levels of lecithin and speed and a higher level of cholesterol, also showed the presence of broken lipid film. Other batches (L-2, L-4, L-5, L-6, L-7, L-8) showed good spherical liposomes.

Batch L-6 was selected as the optimized batch as it also showed the highest entrapment efficiency of 42.6 (0.00)% and also gave an acceptable picture in TEM.

Phase Transition Studies of Liposomes by Differential Scanning Calorimetry

DSC thermograms of blank unloaded liposomal dispersion showed a shift in the melting endotherm of cholesterol from 150.01°C (Fig. 2a) to 125.17°C

(Fig. 3a) and for lecithin from 42.64°C (Fig. 2b) to 72.2°C (Fig. 3a), signifying that all the lipid components interact with each other to a great extent while forming the lipid bilayer.

In the DSC thermogram of salicylic acid liposomal dispersion, Batch L-6, the cholesterol endotherm exhibited a shift form 150.01°C (Fig. 2a) to 133.53°C (Fig. 3b) while the lecithin endotherm was found to be shifted from 42.64°C (Fig. 2b) to 73.44°C (Fig. 3b). Absence of the melting endotherm of salicylic acid suggested significant interaction of salicylic acid with the bilayer structure. The above observation is concordant with the findings of Ladbrooke et al.[15] that interaction of the encapsulated active with the lipid components of liposomes may alter the physicochemical properties of liposomes, which in turn would influence the transfer of the active from the liposomes.

Lamellarity by Nuclear Magnetic Resonance (^{31}P NMR)

The ^{31}P NMR of the salicylic acid liposomes (Batch L-6) was recorded before and after the addition of Mn^{2+}. Before the addition of Mn^{2+}, phosphorus molecules in the phospholipids give characteristic NMR peaks in the bilayer structure. Manganese ions, when added, interact with the outer leaflet of the outermost bilayer and broaden the peak. The reduction in the signal can be correlated to the lamellarity of the vesicles present. Thus, a 50%, reduction in NMR signal means that the liposome preparation is unilamellar, and 25% reduction in the intensity of the original NMR signal means that there are two bilayers in the liposome.[16]

Before the addition of manganese ions (Fig. 4a), the ^{31}P NMR spectra showed a very sharp peak at 2.323 ppm. When manganese ions were added to the salicylic acid liposomes, a notable shift in peak was observed from 2.323 to 3.762 ppm (Fig. 4b). A characteristic broadening in the peak was also observed. The reduction in the NMR signal after addition of manganese ions was found to be 25%, suggesting that the liposomes are bilayered.

Release of Salicylic Acid from Liposomes

Table 3 and Fig. 5 show the mean cumulative percentage release of various batches of salicylic acid liposomes. It was seen that Batches L-2, L-4, L-5, L-7, and L-8 gave a cumulative percentage release of about 90–95% in 6 h. Batch L-6 gave a cumulative release of 85.73% in 6 h and hence was able to control the release of the active for a longer period of time. The release curves obtained were rapid during the first hour, then linear for the following 5 h.

Batches L-1 and L-3 did not show the formation of liposomes in TEM and hence were not subjected to release studies.

Microbiological Studies (Total Microbial Count) of the Salicylic Acid Liposomes

The TMC of the liposomes (Batch L-6) was found to be 20 CFU/mL as determined by the plate count method as described in I.P. 1996.[13]

The CFTA guidelines[17] specify the microbial count to be up to 1000 microorganisms/g or mL.

Thus, the preparation passed the test for TMC.

Optimization Study of Liposomes

The optimization study revealed that as the lecithin concentration was increased, it resulted in a corresponding increase in the entrapment efficiency, whereas a decrease in the lecithin concentration gave a decrease in the entrapment efficiency. Increase in the cholesterol concentration decreased the entrapment efficiency. Thus, higher entrapment efficiency was achieved at a lower cholesterol concentration. This may be observed as increasing cholesterol beyond a certain concentration can disrupt the regular linear structure of the liposomal membrane thereby reducing the entrapment efficiency.[5,14] It was also seen that higher entrapment efficiency was observed at the higher level of speed of rotation (rpm) of Rotovap.

The entrapment efficiency was found to be in the range of 6.256% (± 0.00516) to 42.6% (0.000) with the highest entrapment efficiency of 42.6% (0.000) shown by Batch L-6. Batch L-6 also showed uniform spherical vesicles with a particle size of 0.35 μm as further evaluated by transmission electron microscopy studies. Thus, Batch L-6 was chosen as the optimized batch.

Statistical Evaluation of Factorial Design Data

The experimental data of percentage entrapment efficiency obtained by 2^3 factorial design was tabulated and Yate's treatment was applied to it.

Table 8. Yate's treatment table for % entrapment efficiency.

Expt. no.	Batch no.	% Entrapment efficiency	Column 1	Column 2	Column 3	Column 4 (average effects)	Column 5 (mean squares)
1	L-1	6.256 (±0.00516)	45.566	85.051	—	—	—
A	L-2	39.31 (±0.5477)	39.485	136.205	83.856	20.964	878.97
B	L-3	5.205 (±0.005477)	74.221	62.129	−18.318	4.5795	41.9436
AB	L-5	34.28 (0.00)	61.984	21.727	−4.21	−1.0525	2.2155
C	L-4	31.621 (±0.00408)	33.054	−6.081	51.154	12.7885	327.0914
AC	L-6	42.60 (0.00)	29.075	−12.237	−40.402	−10.1005	204.0402
BC	L-7	25.618 (±0.1918)	10.979	−3.979	−6.156	−1.539	4.7370
ABC	L-8	36.366 (±0.02581)	10.748	−0.231	3.748	0.937	1.75593

Table 9. ANOVA (analysis of variance) table following Yate's treatment of the data originally shown in Table 8.

Factor or interaction	Experiment	Mean squares	F
Lecithin concentration	A	878.97	302.79
Cholesterol concentration	B	41.9436	14.44
Lecithin concentration × cholesterol concentration	AB	2.2155	0.7632
Speed	C	327.0914	112.68
Lecithin concentration × speed	AC	204.0402	70.2905
Cholesterol concentration × speed	BC	4.7370	1.6318
Lecithin concentration × cholesterol concentration × speed	ABC	1.75593	0.6049

Note: Experimental error of the system = 2.90281. Degrees of freedom = 1.

Table 8 shows the data of entrapment efficiency after Yate's treatment. These data were further analyzed statistically by using ANOVA (analysis of variance) to separate the significant terms (factors) from the insignificant terms.

The F-values (Table 9) were calculated. These calculated F values were then assessed by comparing them with the tabulated F values from the table for critical values of the F distribution.

The numerator has 1 degree of freedom and the denominator has 3. Therefore for $p < 0.05$, F should exceed 10.1. Thus, it can be clearly seen that the concentration of lecithin is the most important factor, the next significant factor being the speed of rotation of Rotovap (rpm), followed by the interaction of concentration of lecithin and speed of rotation of Rotovap and then the concentration of cholesterol.

Skin Deposition Studies

The in vitro permeation was calculated in terms of the mean cumulative amount diffused (Q, mg/cm^2) at each sampling point during the 72-h study. The results for both plain salicylic acid dispersion and liposomal salicylic acid dispersion, are shown in Tables 10(a–c).

Student's t-test was applied to the data. As the calculated t-value exceeded the t-critical value at $p < 0.05$, it was evident that cumulative permeation of salicylic acid was significantly greater from plain salicylic acid dispersion than from liposomal salicylic acid dispersion ($p < 0.05$) at all time points. It was thus proved that liposomal entrapment of salicylic acid significantly prolongs the release of salicylic acid across the skin.

The data of mean cumulative amount released (Q, mg/cm^2) vs. square root of time (sec$^{1/2}$) were subjected to linear regression analysis to determine the relationship between these two variables (r).

The value for correlation coefficient (r) for plain salicylic acid dispersion was found to be 0.99250, and liposomal salicylic acid with dispersion was found to be 0.99250, suggesting a linear relationship between Q and $t^{1/2}$ for both the dispersions.

The skin retention (Table 10c) of both the salicylic acid dispersions were studied. It was observed that liposomal salicylic acid dispersion produced higher deposition of salicylic acid in the skin

Table 10a. In vitro permeation studies of plain salicylic acid liposomal dispersion and liposomal salicylic acid dispersion.

		Plain salicylic acid dispersion ($n = 6 \pm$ S.D.)		Liposomal salicylic acid dispersion ($n = 6 \pm$ S.D.)	
Time (h)	Time ($sec^{1/2}$)	Mean cumulative release (mg/cm^2)	% Cumulative (% dose)	Mean cumulative release (mg/cm^2)	% Cumulative (% dose)
1	60.00	0.06413 (± 0.000015)	0.7275 (± 0.00015)	0.0184 (± 0.00027)	0.1633 (± 0.0024)
2	84.85	0.38637(± 0.00227)	4.3894 (± 0.0277)	0.1124 (± 0.00040)	0.9986 (± 0.0035)
3	103.92	0.8000 (± 0.0594)	9.0766 (± 0.0674)	0.2320 (± 0.00034)	2.0599 (± 0.003)
4	120.00	1.2847 (± 0.004482)	14.5758 (± 0.05085)	0.3748 (± 0.00537)	3.3275 (± 0.0477)
5	134.16	1.8685 (± 0.01252)	21.1988 (± 0.14238)	0.55136 (± 0.00502)	4.8949 (± 0.0445)
6	146.94	2.5040 (± 0.009613)	28.4088 (± 0.1090)	0.7368 (± 0.00776)	6.5415 (± 0.0689)
24	293.94	4.2840 (± 0.03445)	48.5787 (± 0.37802)	1.2638 (± 0.01286)	11.220 (± 0.1142)
48	415.69	6.11949 (± 0.0348)	69.4255 (± 0.3957)	1.8027 (± 0.01314)	16.0048 (± 0.1166)
72	509.12	7.9806 (± 0.0350)	90.5386 (± 0.3984)	2.3567 (± 0.01824)	20.922 (± 0.1619)

Note: Thickness of skin = 2.00 mm.
Volume of Franz diffusion cell = 33 mL.
Area available for diffusion = 2.269 cm^2.

Table 10b. Correlation coefficient (r) and t-value for plain salicylic acid dispersion and liposomal salicylic acid dispersion.

Parameters	Plain salicylic acid dispersion	Liposomal salicylic acid dispersion
Correlation coefficient (r)	0.99250	0.99250
t-Value	7.6103	

Table 10c. Percentage of salicylic acid retention in skin after 72 h ($n = 6 \pm$ S.D.).

Plain salicylic acid dispersion	Liposomal salicylic acid dispersion
6.1481 (± 1.13862)%	65.9609 (± 1.1746)%

(65.9609 ± 1.746%) than did plain salicylic acid dispersion (6.1481 ± 1.13862%).

It was thus seen that liposomal entrapment of salicylic acid not only prolonged the release of salicylic acid across the skin but also enhanced the retention of salicylic acid in the skin.

Stability Studies of Liposomes

Visual Appearance and Observations

The changes in the visual appearance are shown in Table 4. It was seen that there was no change in the color of the preparation up to 12 weeks of storage. Slight sedimentation was observed after 12 weeks of storage at room temperature, 8 weeks of storage at 37 and 45°C, whereas the batches stored at 55°C showed a slight liposome sedimentation after 4 weeks of storage and a remarkable sedimentation after 8 weeks of storage.

Particle Size Profiles and Microscopic Observations

Transmission Electron Microscopy

Figure 6a–e depict the TEM of Batch L-6 taken after 12 weeks of storage at different temperatures. The TEM of liposomes stored at 4–5°C (Fig. 6a) showed uniform spherical appearance of liposomes, suggesting that the liposome structure was maintained at 4–5°C (Table 5). The particle size was found to be 0.35 μm at 4–5°C.

At room temperature (Fig. 6b), the formulation showed slight leakage of the active which was seen deposited around the liposome particle. The particle size was 0.3334 μm (Table 5).

The TEM of liposomes stored at 37°C (Fig. 6c) showed distinct disruption of the outer layer of the liposomes leading to increased leakage of the active. The particle size was 0.8261 μm (Table 5).

Figure 6d depicts the TEM of Batch L-6 stored at 45°C after 12 weeks. Greater disruption of the outer layer of the liposome with increased leakage of the active was observed after storage of the liposomes at 45°C. The particle size was 0.9999 μm (Table 5).

The TEM of liposomes stored at 55°C (Fig. 6e) showed disruption of the entire structure of the liposomes with maximum leakage of the active. The particle size increased to 1.16136 μm (Table 5).

It was thus evident from the TEM of the stability studies of the liposomes that maximum stability was observed at refrigeration temperature (4–5°C), wherein the liposomes retained their normal structure and minimum leakage of the active was observed.

Maximum instability of the liposomes was observed at 55°C.

Extent of Leakage of Salicylic Acid from Liposomes upon Storage

For liposomes stored at 4–5°C, extent of leakage was minimal as only 4.01% of the active leaked out after 12 weeks of storage (Table 6 and Fig. 7). At room temperature, 20% leakage of salicylic acid was observed. At 37°C, about 31% of salicylic acid leaked out. Maximum instability was observed at 45 and 55°C showing maximum leakage of salicylic acid of 79.93 and 92.85% respectively.

Thus, from the studies, it was observed that liposomes remained more stable at refrigeration temperature.

Microbiological Studies

Total microbial count of liposomes after storage for 12 weeks at room temperature:

TMC of the liposomes (Batch L-6) was found to be 80 CFU/mL as determined by the plate count method as described in I.P. 1996.[13]

The TMC showed an increase after storage but was within the limits of the CFTA guidelines.[17] The preparation thus passed the test for TMC after stability studies.

CONCLUSION

The conventional thin film hydration technique was found to be simple and suitable for laboratory-scale preparation. The liposomes obtained by this method showed the presence of bilayered vesicles as confirmed by the ^{31}P NMR studies. The liposomes were found to be in the range of 0.2–0.8276 μm as shown in the TEM photomicrographs. The optimized batch gave a good controlled release of salicylic acid from liposomes as shown by the in vitro release profiles. The liposomal dispersions stored at 4–5°C retained salicylic acid to a greater extent than that stored at any other higher temperatures.

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