Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 5, 2015

Original Article

EVALUATING THE IMPACT OF PREPARATION CONDITIONS AND FORMULATION ON THE ACCELERATED STABILITY OF TRETINOIN LOADED LIPOSOMES PREPARED BY HEATING METHOD

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Received: 02 Feb 2015 Revised and Accepted: 01 Mar 2015

ABSTRACT

Objective: The aim of this study was to prepare stable all-trans retinoic acid or tretinoin (RA) loaded liposome formulation with high encapsulation efficiency intended for immediate application on the skin using the simple preparation method.

Methods: Formulas were prepared by heating method. The effect of formulation variables on liposome properties was investigated. Dynamic Light Scattering Process (DLS) and the electrophoretic mobility process were used to determine the mean size and zeta potential, respectively. The encapsulation efficiency % was determined spectrophotometrically. Six liposome formulas (Fs) were prepared. Three types of phospholipids and two ratios of cholesterol were used. Sodium cholesteryl sulfate (SCS) was added to all formulas except formula 1. Six formulas and topical ethanolic solution of RA was stored at accelerated storage conditions for six months. EE% was determined by validated H. P. L. C method in the stage of the stability study.

Results: pH=6.5 showed the highest EE%. Substantial decrease in zeta potential and EE% was noticed by increasing the ionic strength. SCS showed a positive effect on the stability of liposomes. Hydrogenated soybean phosphatidylcholine (HSPC) showed the best results on the stability of liposomes and RA. The leakage of RA from liposomes was not observed when the cholesterol was added to the formulas.

Conclusion: The phospholipid that has a phase transition temperature (T_m) above the storage temperature showed the best stability with time and prevented the leakage of the active substance without needing to add cholesterol. It is necessary to add charge inducing substance to the formula to improve its stability.

Keywords: Heating method, Ionic strength, Liposome, Tretinoin, Zeta potential, Accelerated stability

INTRODUCTION

Tretinoin (all-trans retinoic acid) is one of the retinoids that are involved in several biological processes, particularly vision, cell proliferation and differentiation [1]. In the pharmaceutical field, all-trans retinoic acid or tretinoin (RA) is widely used in the treatment of various dermatological conditions that include acne vulgaris, psoriasis, photodamaged skin and other skin disorders [2]. Retinoids involving RA also showed beneficial efficacy and good safety profiles as therapeutic anti-ageing agents [3]. Due to several side effects when systemically administered [4], all-trans retinoic acid is almost exclusively topically employed, resulting in reduced side effects if any RA being absorbed systematically [3]. However, even its topical use is limited by several disadvantages, such as skin irritation, very low water solubility and high instability in the presence of air, light and heat [4]. Liposomes are spherical vesicles with an aqueous cavity at their center and encasing envelope which is made up of a varying number of bimolecular sheets (lamellae) mainly composed of phospholipids [5]. The similarity between most of liposome components and cutaneous lipids has made liposomes one of the prime carriers of dermatological ingredients [5]. The use of liposomes has provided for a higher concentration of drugs in deeper layers of the skin and a reduction in percutaneous absorption and unwanted side-effects [6]. Different investigators reported an increased skin accumulation of RA in-vitro and a reduced irritancy in-vivo after treatment with these carriers. It was observed that the maximum comedolytic activity of RA is reached at a concentration of five to ten times lower when RA is incorporated into liposomes, compared to the conventional alcoholic gels [7]. Unsaturated phosphatidylcholine supports skin regeneration, anti-ageing, prevention of acne and penetration of other active agents such as vitamins and their derivatives into the skin [8]. Several authors have studied the tretinoin loaded liposome formulations to prevent its fast degradation. Inclusion of RA in liposomes has been reported to protect it against photodegradation [1]. It was demonstrated that RA can be incorporated in high amounts in both liposomes and niosomes [9], which are also able to reduce the photodegradation rate of the drug [10].

The objective of this research was to prepare tretinoin loaded liposome suspension for immediate topical application, that showed high encapsulation efficiency and was stable with time at accelerated storage conditions using simple preparation method.

MATERIALS AND METHODS

Tretinoin (all-trans retinoic acid) was purchased from Sigma-Aldrich (China). L-α-phosphatidylcholine (from soybean, type IV-S ≥30% (TLC)) (SPC), L-α-phosphatidylcholine, hydrogenated (HSPC) and sodium cholesteryl sulfate were purchased from Sigma-Aldrich (USA), 1, 2-Dimyristoyl-rac-glycero-3-phosphocholine was supplied by Sigma-Aldrich (Switzerland). Cholesterol was supplied by Sigma-Aldrich (Germany). Buthylhydroxytoluene (BHT), potassium di-hydrogen phosphate and ethanol were supplied from Panreac (E. U, Barcelona-Spain). Triton-X 100 LR was supplied by Rasayan laboratory-chem limited (Mumbai-India). Glycerol was supplied by BDH Laboratory (England), propylene glycol was provided from Merck, prolabo (CE) and isopropyl alcohol was supplied from Surechem products LTD-Needham market Suffolk (England). HPLC grade acetonitrile was provided from Scharlab (Spain) and trifluroacetic acid (TFA) was provided from Sigma-Aldrich (USA). Double distilled and de-ionized water was used.

Preparation of tretinoin-loaded liposomes

The tretinoin loaded liposomes were prepared using the heating method [11]. SPC: 100 mg and BHT: 5 mg were hydrated in distilled and de-ionised water (9 ml), in presence of glycerol 3% of the final volume. The mixture was rotated by a magnetic stirrer at speed 1300 rpm and heated up to

100 °C in a water bath. It was left at this temperature for 1 h. Then the water bath was switched off, but the rotation was continued. The temperature reduces gradually. When the temperature has reached to 30 °C, active substance RA: 2.5 mg was added. The rotation was stopped after 10 min of adding the active substance. The final volume was adjusted to 10 ml. The resulting liposome suspension was sonicated for 15 min for size homogenizing. Every preparation was repeated three times. The optimization of the formulation in each step was evaluated by the determination of the encapsulation efficiency, mean size, PdI and zeta potential.

Determination of phospholipid quantity effect

Different amounts of soybean phosphatidylcholine SPC: 100, 150, 200 mg were used to prepare 10 ml of RA-loaded liposome suspension. All other constituents of the formulation were unchanged. The encapsulation efficiency, mean size, polydispersity index and zeta potential were measured before and after 15 min sonication.

Determination of water-miscible solvent type effect

Two types of solvents were tested: glycerol and propylene glycol. 3% v/v of each solvent was used. The third formulation was prepared without adding any solvent to the mixture. All other constituents of the formulation were unchanged. The encapsulation efficiency, mean size, polydispersity index and zeta potential were measured before and after 15 min sonication.

Determination of the active substance adding temperature effect

The formulations were prepared without changes in the constituents and their quantities. SPC: 100 mg and BHT: 5 mg were hydrated in distilled and de-ionised water 9 ml, but active substance RA: 2.5 mg was added at 30 °C, 45 °C and 65 °C, respectively. The changes in an encapsulation efficiency, mean size, polydispersity index and zeta potential were measured before and after 15 min sonication.

Determination of rotating speed effect

The formulations were prepared without any change in the constituents and their quantities, but two different rotating speed was tested (1300 rpm, 600 rpm). The encapsulation efficiency, mean size, polydispersity index and zeta potential were measured before and after 15 min sonication.

Determination of sonication time effect

The formulations were prepared without any change in the constituents and their quantities. Various sonication time was tested. The encapsulation efficiency, mean size, polydispersity index and zeta potential were measured immediately after preparation and after: 5, 10, 15, 30 and 60 min of sonication.

Determination of pH phosphate buffer effect

The same constituents SPC: 100 mg and BHT: 5 mg were hydrated in phosphate buffer at three different pH (5.8, 6.5 and 8) and ionic strength 0.05 M, in presence of glycerol 3% of the final volume. The same steps were continued. The formulations were compared together. The encapsulation efficiency, mean size, polydispersity index, zeta potential and pH were measured before and after 15 min sonication. The homogenized formulations were left at a room temperature for 4, 12 and 30 d. The measuring was repeated at these points.

Determination of ionic strength effect

The same constituents SPC: 100 mg and BHT: 5 mg were hydrated in phosphate buffer at three different ionic strengths (0.025, 0.05 and 0.1 M) and pH 6.5, in presence of glycerol 3% of the final volume. The same steps were continued. The formulations were compared together. The encapsulation efficiency, mean size, polydispersity index, zeta potential and pH were measured before and after 15 min sonication. The homogenized formulations were left at a room temperature for 20 d. The measuring was repeated at this point.

Preparation of final formulas

The optimal conditions resulted from the former studies were used to prepare the final formulas. The formulas 1 and 2 were prepared to determine the effect of adding negative SCS on the stability of liposomes and the active substance. Formula 1 was prepared as follows: SPC: 300 mg and BHT: 15 mg were hydrated in phosphate buffer pH=6.5 and ionic strength 0.025 M (27 ml), in presence of glycerol 3% of the final volume. The mixture was rotated by a magnetic stirrer at speed 1300 rpm and heated up to 100 °C in a water bath. It was left at this temperature for 1 h. Then the water bath was switched off, but the rotation was continued. The temperature reduces gradually. When the temperature has reached to 30 °C, active substance RA: 7.5 mg was added. The rotation was stopped after 10 min of adding the active substance. The final volume was adjusted to 30 ml. The resulting liposomal suspension was sonicated for 15 min for size homogenizing. Every preparation was repeated three times. Formula 2 was prepared as follows: 12 mg of sodium cholesteryl sulfate was added to the other constituents in the formula 1 and the same preparation steps were followed. Formulas 2, 3 and 4 were prepared to study the effect of phospholipid types on the stability of liposomes and the active substance. Formula 3 was prepared as follows: SPC in the formula 2 was substituted by HSPC in the formula 3 without any change in the other constituents. The same preparation steps were followed. Formula 4 was prepared as follows: SPC in the formula 2 was substituted by DMPC in the formula 4 without any change in the other constituents. The same preparation steps were followed. Formulas 5 and 6 were prepared to study the effect of cholesterol on the stability of liposomes and the active substance. 20% and 40% of SPC in the formula 2 were substituted by cholesterol in the formulas 5 and 6, respectively, without any change in the other constituents. The same preparation steps were followed. To prepare the topical ethanolic solution of tretinoin formula 7 was prepared as follows: 7.5 mg of tretinoin and 15 mg of BHT were dissolved in 18 ml ethanol 96%. Then 7.5 g of propylene glycol was added. The final volume was adjusted to 10 ml using distilled and de-ionised water.

Study the accelerated stability of final formulas

According to ICH Q1A (R2) the final formulas (F1 to F7) were stored at 40 °C ± 2 °C/75% RH±5% RH for 6 mo using cabinet (Binder/Germany) [12]. Encapsulation efficiency, mean size, PdI, zeta potential and pH were determined in the following time points (0, 1, 2, 3 and 6 mo). The total percentage of active substance was determined in the following time points (0, 3 and 6 mo).

Liposomes characterization

Mean hydrodynamic diameter and zeta potential

Mean hydrodynamic diameter (Z-average) of tretinoin loaded liposomes and zeta potential (Zp) was determined using a Malvern Zetasizer Nano-series (Nano-Zsm Malvern Instruments, Malvern, UK). The Zetasizer Nano-series performs size measurements using a process called Dynamic Light Scattering (DLS), which measures Brownian motion and relates it to the size of particles. Polydispersity index was also obtained. This index ranges from 0 to 1; a higher value indicates a less homogeneous liposomes size distribution. Zeta potential (refers to the stability of suspension with time) was determined by measuring the velocity of the particles in an electric field, which was referred to as electrophoretic mobility using Laser Doppler Velocimetry (LDV) and then applying the Henry equation. All measurements were performed at 25 ˚C and detector angle 173° without diluting; each data value is an average of three measurements.

Encapsulation efficiency

The stage of optimization the preparation conditions

During the stage of determining the optimal preparation conditions, the drug encapsulation efficiency in liposomes was determined spectrophotometrically, using (spectrophotometer-V-650, Japan) according to USP30. Stock solution in acidified isopropyl alcohol (prepared by mixing 1 ml of 0.01 N hydrochloric acid with isopropyl alcohol to make 1000 ml of solution) was prepared with a concentration of 1 mg/ml. Concentrations within a range 0.15-30 µg/ml were prepared by an appropriate dilution with acidified isopropyl alcohol of stock solution and used to set up the calibration curve. The absorbance was determined at the wavelength of

maximum absorbance 352 nm. Acidified isopropyl alcohol was used as the blank. The linearity was established within this range. The equation of the regression line was y=0.141x-0.0243, R^2=0.9997. Total tretinoin concentration (C_T) was determined by diluting 1 ml of liposomal suspension with triton-X 100 (5%) to 10 ml and sonicating for 15 min, this solution was further diluted 1 ml to 5 ml with acidified isopropyl alcohol and then the absorbance at 532 nm was measured.

For determining the concentration of unentrapped drug (free drug C_F), 1 ml of suspension was ultracentrifugated at 40 000 rpm and 25 °C for 30 min (when suspension was ultracentrifugated at two different temperatures 4 °C and 25 °C, the difference was negligible) using a CP 80WX Himac preparative ultracentrifuge, Hitachi, Japan. The supernatant was removed and diluted to 10 ml with acidified isopropyl alcohol and then the absorbance at 352 nm was measured.

The encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency } \% = \frac{C_T - C_F}{C_T} \times 100$$

The stage of accelerated stability study

During the stage of accelerated stability study, EE% was determined by H. P. L. C analytical method. HPLC separation was performed with Shimadzu Liquid chromatographer (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a SPD-M20A photodiode array detector, LC-20AT pump system, CTO-20A oven, and SIL-20A auto sampler. Quantitative measurement of tretinoin content was done at 354 nm. The tretinoin was separated on a silica gel column (EC 250/4.6 Nucleodur 100-5 C18, Macherey-Nagel, Germany).

A gradient elution was achieved with an initial run of acetonitrile: TFA 0.01% in the ratio of 70:30 (v/v) for 24 min, which was then changed to 65:35 (v/v) for next 4 min and finally returned back to the same ratio of 70:30 (v/v) for 4 min. The ratio of 70:30 (v/v) was stayed for additional 5 min to equilibrate the column. Other HPLC parameters were: flow rate 1 ml/min, injection volume 20 µl and oven temperature 30 °C.

The calibration of peak area versus tretinoin concentration was linear in the concentration range of (3.5–30 µg/ml) (R^2= 0.9998). Total tretinoin concentration (C_T) was determined by diluting 1 ml of liposomal suspension with triton-X 100 (10%) to 5 ml and sonicating for 15 min, this solution was further diluted 1 ml to 5 ml with acetonitrile and then the absorbance at 354 nm was measured. For determining the concentration of unentrapped drug (free drug C_F), 1 ml of suspension was ultracentrifugated at 40 000 rpm and 25 °C for 30 min (when suspension was ultracentrifugated at two different temperatures 4 °C and 25 °C, the difference was negligible) using a CP 80WX Himac preparative ultracentrifuge, Hitachi, Japan. The supernatant was removed and diluted to 10 ml with acetonitrile and then the absorbance at 354 nm was measured. The encapsulation efficiency was calculated as mentioned before. Injections, in triplicate, were done at each concentration for standards and samples. The analytical method was validated as usually required.

Determination of pH

pH was determined in the following time points (0, 1, 2, 3 and 6 mo) using pH measurement (Crison/Spain).

RESULTS AND DISCUSSION

Preparation of tretinoin-loaded liposomes

Determination of phospholipid quantity effect

Neutral liposomes composed of soybean phosphatidylcholine showed a high negative zeta potential -68.1 mV in de-ionised water, which may be attributed to the orientation of the lipid hydrophilic head group. It was thought that the phosphatidyl groups have located at the outer portion of the head group region when phospholipid was dispersed in distilled and de-ionised water, consequently the liposomal particles have exhibited the negative surface charge. Mosharraf and coworkers also noticed that neutral liposomes composed of egg phosphatidylcholine and dipalmitoylphosphatidylcholine had showed a high negative zeta potential in de-ionized water [13].

Before homogenizing, when 100 mg of the SPC has used, tretinoin-loaded liposomes showed the smallest particle size, the highest zeta potential, the highest EE% and the most homogeneous size distribution (PdI was the smallest) as it is shown in table (1). By increasing the amount of the phospholipid, the particle size increased too. A little change in zeta potential was observed.

After homogenizing, the size distribution became more homogeneous for all amounts. The mean size and zeta potential changed. This change in zeta potential value was expected to occur simultaneously with the total surface area change of suspended particles, in comparison with before sonication. The high lipophilicity of tretinoin may increase the tendency of the active substance to encapsulate inside the phospholipid bilayer membrane.

This may explain the high encapsulation efficiency of tretinoin. Little differences in EE% in the three used amounts were observed. After sonication it was noticed that EE% decreased, but all values remained close to each other. 100 mg amount showed the best homogeneous (PdI was the lowest) and the highest zeta potential (liposomes are more stable with time). The stability seems to be the best in a range of about (100–300 nm) [8] and the mean size of liposomes that were prepared using the 100 mg amount of the SPC was inside this range.

Besides, phosphatidylcholine need not be applied in high concentrations due to the experience showed that the formulations were stable at lower amounts and there was the cumulative effect in horny layer with repeated application of phosphatidylcholine [8]. For these reasons, 100 mg of the SPC was chosen for the following experiments.

Table 1: The effect of phospholipid quantity on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

Phospholipid amount	**100 mg**	**150 mg**	**200 mg**
sample	**LP_1**	**LP_2**	**LP_3**
Before sonication			
Mean diameter (nm)±SD[a]	364.7±2.97	403.6±2.41	508.6±43.34
PdI±SD[a]	0.561±0.003	0.984±0.015	0.766±0.21
Zeta potential±SD[a]	-61.7±0.81	-58.7±3.52	-57.9±1.15
EE%±SD[a]	92.84±0.02	88.34±0.06	90.829±0.023
After 15 min sonication			
Mean diameter (nm)±SD[a]	194.56±16.74	415.95±6.15	242.75±100.05
PdI±SD[a]	0.459±0.044	0.6745±0.08	0.532±0.018
Zeta potential±SD[a]	-63.2±2.83	-53.1±2.22	-60.2±10.4
EE%±SD[a]	87.27±3.38	90.24±1.54	86.16±3.75

[a]n = 3; SD: standard deviation between the three assays.

Determination of water-miscible solvent type effect

The effect of water-miscible solvent type on the encapsulation efficiency, liposome size, polydispersity index and zeta potential is presented in table (2).

Before homogenization, It was noticed that the liposomes prepared with glycerol showed the smallest particle size and the most homogeneous size distribution. This may be attributed to the higher viscosity of this solvent. The elevating of temperature during preparation increased the movement of forming particles, consequently the probability of their collision and interfusing together increased, but increasing the viscosity of medium simultaneously with gradual decreasing of temperature prevented the movement of particles and decreased their ability to collide and interfuse together. When the viscosity of the medium has increased, a little decrease in zeta potential was observed. It was expected that the weak acidity of glycerol and propylene glycol may have caused protonation of some of the surface phosphatidyl groups responsible for surface charge, so zeta potential was the highest when only water was used. A little difference in the EE% in the three preparations was noticed. The EE% was the highest when only water was used. It was thought that the viscosity of the medium may have showed estopping to movement the suspended active substance toward particles to encapsulate inside the phospholipid bilayer membrane. After homogenizing, the size distribution became more homogeneous for all formulations and the mean size was the highest with propylene glycol.

In addition, EE% showed a little decrease, but stayed related to all formulations. The formulation that was prepared by glycerol showed the most homogeneous size distribution and high zeta potential. The mean size of obtaining particles was inside the best stable range (100-300 nm) [8]. Glycerol showed the largest estopping to interfuse of the suspended particles. For these reasons, glycerol was chosen for preparation the following formulations.

Table 2: The effect of water miscible solvent type on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

Sample	Glycero l 3%	Propylene glyco l 3%	Only water
	LP_1	LP_4	LP_5
Before sonication			
Mean diameter (nm)±SD[a]	364.7±2.97	393.4±19.9	608.3±14.03
PdI±SD[a]	0.561±0.003	0.808±0.135	0.978±0.021
Zeta potential±SD[a]	-61.7±0.81	-63.4±0.56	-67.2±0.96
EE%±SD[a]	92.84±0.02	94.67±0.626	95.06±0.3
After 15 min sonication			
Mean diameter (nm)±SD[a]	194.56±16.74	402.1±9.47	332.3±6.92
PdI±SD[a]	0.459±0.044	0.581±0.025	0.573±0.016
Zeta potential±SD[a]	-63.2±2.83	-55±1.01	-65.7±0.808
EE%±SD[a]	87.27±3.38	90.1278±0.99	89.0784±0.4

[a]n = 3; SD: standard deviation between the three assays.

Determination of the active substance adding temperature effect

From the results that are mentioned in the table (3), it was noticed that when the active substance was added at a higher temperature, the mean size has increased and size distribution has become less homogeneous. Zeta potential values were close to each other and EE% was almost the same in the three different temperatures. After homogenizing, the mean size decreased and size distribution became more homogeneous. A little change in zeta potential was noticed, but all values stayed related. EE% showed a little decrease, but no significant difference was observed in the three formulations.

Tretinoin is sensitive to heat [4]. The lowest temperature (30 °C) showed both the smallest mean size and PdI. Beside, there was no significant difference in zeta potential and EE% in the three temperatures (30 °C, 45 °C and 65 °C). Hence, (30 °C) was retained for further studies.

Table 3: The effect of the active substance adding temperature on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

Sample	30 °C	45 °C	65 °C
	LP_1	LP_6	LP_7
Before sonication			
Mean diameter (nm)±SD[a]	364.7±2.67	406.5±3.8	471.1±11.83
PdI±SD[a]	0.561±0.003	0.582±0.013	0.625±0.046
Zeta potential±SD[a]	-61.7±0.81	-64.4±0.802	-65.5±2.09
EE%±SD[a]	92.84±0.02	91.88±0.06	92.968±0.07
After 15 min sonication			
Mean diameter (nm)±SD[a]	194.56±16.74	376.7±27.2	278.2±6.88
PdI±SD[a]	0.459±0.044	0.525±0.001	0.473±0.034
Zeta potential±SD[a]	-63.2±2.83	-56.95±0.05	-60.1±4.25
EE%±SD[a]	87.27±3.38	86.62±4.56	86.24±2.81

[a] n = 3; SD: standard deviation between the three assays.

Determination of rotating speed effect

As it is shown in table (4) the smallest particle size of tretinoin-loaded liposomes (364.7 nm) and the highest EE% were obtained by rotating speed (1300 rpm). When the rotating speed has increased, a significant decrease in mean size was noticed. It can be concluded that the increasing of rotating speed led to more potent fractionalizing of forming particles. Consequently, the size of resulting liposomes decreased. A little increase in EE% was observed by increasing the rotating speed. This may be attributed to increase the total surface area of the resulting particles, which were in contiguity with the active substance. Zeta potential and PdI were related in the both speeds. After sonication, the mean size decreased simultaneously with a little increase in zeta potential and EE%

decreased almost at the same rate in both speeds. PdI became smaller (size distribution became more homogeneous) with (1300 rpm), whereas it became larger with (600 rpm). Because of (1300 rpm) showed the highest EE% and the smallest mean size. Besides, there was no significant difference in zeta potential between the two speeds. Hence, (1300 rpm) was retained for further studies.

Table 4: The effect of rotating speed on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

	1300 rpm	600 rpm
Sample	**LP_1**	**LP_8**
Before sonication		
Mean diameter (nm)±SD[a]	364.7±2.97	1005±63.5
PdI±SD[a]	0.561±0.003	0.539±0.026
Zeta potential±SD[a]	-61.7±0.81	-58.6±2.57
EE%±SD[a]	92.84±0.02	89.059±1.03
After 15 min sonication		
Mean diameter (nm)±SD[a]	194.56±16.74	398.3±17.5
PdI±SD[a]	0.459±0.044	0.784±0.198
Zeta potential±SD[a]	-63.2±2.83	65.95±10.75-
EE%±SD[a]	87.27±3.38	83.25±3.18

[a]n = 3; SD: standard deviation between the three assays.

Table 5: The effect of sonication time on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

	Without sonication	5 min sonication	15 min sonication	30 min sonication	60 min sonication
sample	**LP_1**	**LP_9**	**LP_{10}**	**LP_{11}**	**LP_{12}**
Mean diameter (nm)±SD[a]	364.7±2.97	268.35±100.25	194.56±16.74	161.23±12.68	151.5±1.96
PdI±SD[a]	0.561±0.003	0.499±0.0525	0.459±0.044	0.438±0.027	0.397±0.012
Zeta potential±SD[a]	-61.7±0.81	-62.45±6.15	-63.2±2.83	-69.4±2.454	-70.1±2.7
EE%±SD[a]	92.84±0.02	89.06±2.5	87.27±3.38	78.71±2.67	75.18±3.25

[a] n = 3; SD: standard deviation between the three assays.

Table 6: The effect of pH-phosphate buffer on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

Sample	**5.8**	**6.5**	**8**
	LP_{13}	**LP_{14}**	**LP_{15}**
Before sonication			
Mean diameter (nm)±SD[a]	254.7±6.48	319.75±16.35	270.9±11.43
PdI±SD[a]	0.474±0.013	0.595±0.082	0.563±0.119
Zeta potential±SD[a]	-26.9±1.82	-28.1±0.379	-30.3±0.416
EE%±SD[a]	40.92±0.56	69.31±0.38	55.16±0.113
pH	5.8	6.5	8
After 15 min sonication			
Mean diameter (nm)±SD[a]	237.65±10.45	220.9±6.89	89.21±0.217
PdI±SD[a]	0.456±0.017	0.431±0.008	0.214±0.002
Zeta potential±SD[a]	-25.7±2.95	-26.2±0.321	-28.8±1.83
EE%±SD[a]	35.38±0.31	64.64±0.132	29.72±0.119
pH	5.8	6.52	7.98
After 4 d at room temperature			
Mean diameter (nm)±SD[a]	259.7±1.05	266.8±6.43	117.6±3.66
PdI±SD[a]	0.273±0.017	0.444±0.028	0.289±0.017
Zeta potential±SD[a]	-21.4±3.54	-28.7±0.51	-26.4±1.05
EE%±SD[a]	42.88±0.423	62.35±0.019	29.752±0.119
pH	5.31	6.36	7.69
After 12 d at room temperature			
Mean diameter (nm)±SD[a]	-	-	-
PdI±SD[a]	-	-	-
Zeta potential±SD[a]	-	-	-
EE%±SD[a]	-	-	-
pH	4.22	6.11	7.02
After 30 d at room temperature			
Mean diameter (nm)±SD[a]	477.9±29.86	227.2±1.45	182.3±1.33
PdI±SD[a]	0.415±0.047	0.162±0.027	0.182±0.03
Zeta potential±SD[a]	-11.9±1.05	-25.7±0.557	-23.7±1.35
EE%±SD[a]	ND	ND	ND
pH	3.86	5.9	6.85

[a] n = 3; SD: standard deviation between the three assays.

Determination of sonication time effect

When the time of sonication increased, both the mean size and PdI decreased simultaneously with an increase in zeta potential. The increase in sonication time showed a negative effect on the encapsulation efficiency. By increasing the time of sonication, active substance leaked from liposomes and encapsulation efficiency decreased. The results are presented in table (5). Although the smallest particle size, the most homogeneous size distribution and the highest zeta potential were obtained with 60 min of sonication, but encapsulation efficiency decreased relatively very much when the time of sonication exceeded 15 min. The sonication time 15 min showed relatively a little decrease in EE% and high zeta potential. The mean size of obtaining particles was inside the best stable range (100-300 nm) [8] and PdI became smaller. For these reasons, further studies were done with 15 min sonication.

Determination of pH phosphate buffer effect

Liposomal suspension was prepared at three different pH and ionic strength 0.05 M to study the effect of pH on the hydrolysis of phospholipid. pH was measured immediately after preparation and after 4, 12 and 30 d. Also mean diameter, PdI, zeta potential and EE% were measured as it is shown in table (6). The value of pH= 6.5 showed the lowest decrease in pH with time and the highest EE%. In an aqueous liposome dispersion, the liposomal phospholipids can hydrolyse to free fatty acids and 2-acyl and 1-acyl lysophospholipids [14]. Further hydrolysis of both lysophospholipids results in glycero phospho compounds [14]. An increasing of releasing free fatty acids with time caused the decrease in pH. We assumed that at acidic pH= 5.8 phosphatidyl groups in neutral phospholipids may be protonated, consequently the positive charge that resulted from choline groups led to the appearing of the repulsion force between adjacent hydrophilic head groups in the same particle and increasing the spaces between them, so the ends of lipophilic hydrocarbon chains became more exposed to the aqueous interface. This may explain the highest change in pH at pH= 5.8. The hydrocarbon chains may become more overlapping together simultaneously with decrease the lipophilic bilayer membrane thickness of liposomes to reduce the exposure them to the aqueous medium. This may explain decrease the EE% of the lipophilic active substance. At alkaline pH= 8 it was expected that hydroxyl ions may connect with positive choline groups, consequently repulsion force between adjacent head groups in the same particle appeared as it was mentioned before, whereas it was thought that at pH= 6.5 the nearest to neutralization the spaces between the hydrophilic head groups in phospholipid were smaller, subsequently exposuring the hydrocarbon chains to the aqueous medium became lower and the thickness of bilayer membrane of liposome became larger. This may explain the highest EE% and the lowest change in pH with time. The increasing of hydrocarbon chains overlapping together in both acidic and alkaline pH may have affected the suspended particle size, which was smaller at both pH= 5.8 and 8 in comparison with pH= 6.5. Immediately after preparing the three formulations, little differences in zeta potential values were observed. Zeta potential was the lowest with pH= 5.8 and showed the highest change with time simultaneously with interfusing the suspended particles and increasing the mean size, whereas pH= 6.5 showed the lowest change in both zeta potential and mean size with time. pH= 6.5 showed the lowest change in pH with time and the highest EE%. No significant change in both zeta potential and mean size was noticed. Hence, pH= 6.5 was retained for further experiments.

Determination of ionic strength effect

For evaluating the effect of ionic strength on liposomal suspension characteristics, particularly zeta potential and EE%, liposomal suspension, was prepared at three different ionic strengths and pH= 6.5 as it is shown in table (7). Substantial decrease in zeta potential was noticed by increasing the ionic strength as it is shown in fig. 1. This result was similar to that reported by Makino *et al.* [15], who thought that the direction of the hydrophilic head group of phospholipid was sensitive to the temperature and ionic strength of the medium, so at low ionic strengths, the phosphatidyl groups located at the outer portion of the head group region and by increasing the ionic strength the choline groups approached the outer region of the bilayer surface while the phosphatidyl groups hid behind the surface, subsequently, the negative surface charge decreased. The lowest ionic strength showed the highest EE% and vice versa as it is shown in fig. 1. Increasing the ionic strength led to an increase in the polarity of the aqueous medium. Consequently, overlapping of non polar hydrocarbon chains may increase, thereby, the thickness of the lipophilic bilayer membrane, which the lipophilic active substance encapsulated inside it decreased and EE% decreased too. After homogenizing, the mean size and EE% decreased and the size distribution became more homogeneous. With time and by increasing the ionic strength, obvious increase in the mean size was observed simultaneously with the decrease of zeta potential. With time pH showed a little change in the highest ionic strength, whereas the change became more obvious by decreasing the ionic strength. The lowest ionic strength 0.025 M showed both the highest EE% and zeta potential and the mean size was relatively stable with time. For these reasons, it was retained for further experiments.

Table 7: The effect of ionic strength on the mean size, polydispersity index, zeta potential and encapsulation efficiency of RA loaded liposomes

Sample	0.025 M	0.05 M	0.1 M
	LP_{16}	LP_{14}	LP_{17}
Before sonication			
Mean diameter (nm)±SD[a]	353.7±10.75	319.75±16.35	331.4±2.71
PdI±SD[a]	0.57±0.038	0.595±0.08	0.48±0.014
Zeta potential±SD[a]	-46.2±3.7	-28.1±0.379	-19.2±0.917
EE%±SD[a]	89.67±0.154	69.31±0.38	51.46±0.15
pH	6.5	6.5	6.5
After 15 min sonication			
Mean diameter (nm)±SD[a]	190.7±0.058	220.9±6.89	187.4±1.08
PdI±SD[a]	0.399±0.02	0.431±0.008	0.282±0.035
Zeta potential±SD[a]	-45.4±4.03	-26.2±0.321	-18.2±0.51
EE%±SD[a]	79.98±0.044	64.64±0.132	25.74±0.17
pH	6.5	6.52	6.5
After 20 d at room temperature			
Mean diameter (nm)±SD[a]	204.1±3.12	284.9±5.024	531.55±17.95
PdI±SD[a]	0.203±0.01	0.153±0.013	0.262±0.016
Zeta potential±SD[a]	-33.2±5.7	-25.7±0.61	-8.43±1.05
EE%±SD[a]	86.33±0.86	49.51±2.37	46.85±0.86
pH	5.58	6.00	6.3

[a] n = 3; SD: standard deviation between the three assays.

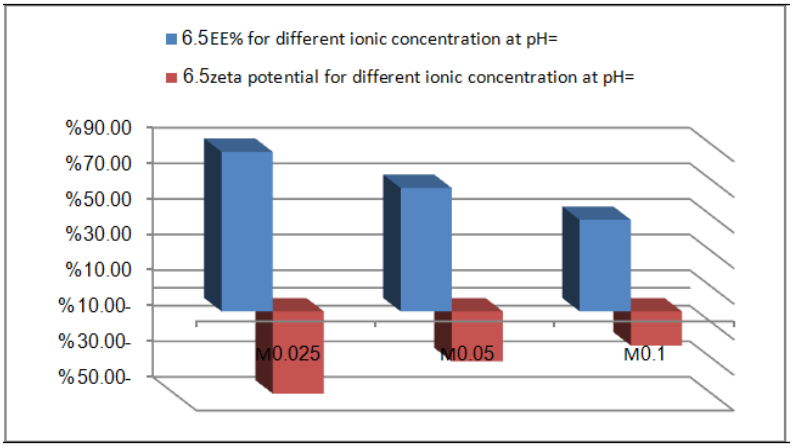

Fig. 1: Encapsulation efficiency% and zeta potential at different ionic strengths and pH= 6.5

Study of the accelerated stability of final formulas

pH: From table (8) it was observed that the related change in pH was observed with formulas 1 and 2. When sodium cholesteryl sulfate was added to the formula, negligible effect on the change of pH with time was observed. The change in pH was less noticeable with both formulas 3 and 4 in comparison with formula 2. By increasing the ratio of cholesterol, the change in pH became less noticeable. This may be attributed to decrease the total amount of phospholipid that was exposure to hydrolysis.

Mean diameter and zeta potential

It was observed that the zeta potential decreased simultaneously with the decrease of pH as it is shown in table (8). Almost all formulas showed related changes in the mean size during the first three months, but the fusion rate increased after that. It was observed that adding sodium cholesteryl sulfate to the formula 2 improved its stability with time in comparison with formula 1, which separated into two layers after six months. Although formula 2 showed aggregation after six months.

Table 8: Accelerated stability study of the final formulas

Formula	Mean diameter (nm)±SD[a]	PdI±SD[a]	Zeta potential ±SD[a]	Total percentage %±SD[a]	EE%±SD[a]	pH	Time point (month)
1	202.1±23.70	0.381±0.033	-46.9±1.2	100±0.04	88.19±0.36	6.50	0
	249.1±2.56	0.418±0.028	-30.2±0.72	-	74.78±0.55	5.78	1
	254.4±5.69	0.383±0.030	-28.1±1	-	86.12±0.73	5.04	2
	249.8±10.71	0.343±0.007	-26.9±2.7	93.59±0.79	87.17±0.11	4.74	3
	Two separated layers were formed						6
	157.9±8.31	0.401±0.074	-53.0±1.60	100±0.34	82.76±0.36	6.50	0
2	204.9±2.43	0.339±0.013	-40.4±1.65	-	77.55±0.77	6.01	1
	200.4±2.67	0.302±0.021	-27.8±1.12	-	85.30±0.58	5.06	2
	217.3±4.61	0.288±0.020	-25.2±1.81	96.10±0.26	88.03±0.41	4.56	3
	515.5±38.68	0.546±0.063	-16.2±1.49	91.65±0.29	92.32±0.06	4.04	6
3	200±3.46	0.621±0.027	-39.1±2.24	100±0.36	81.67±0.94	6.50	0
	220.2±3.56	0.657±0.007	-28.3±2.18	-	82.61±0.11	6.32	1
	268.5±7.59	0.695±0.030	-24.6±1.64	-	83.44±0.45	6.13	2
	315.1±24.47	0.719±0.122	-20.5±2.44	94.51±0.20	86.83±0.25	5.93	3
	281.4±10.29	0.682±0.025	-21.1±0.86	92.75±0.35	90.69±0.17	5.90	6
4	175.2±26.86	0.407±0.094	-41.3±2.43	100±0.81	56.52±0.81	6.50	0
	235.3±6.11	0.627±0.031	-31.2±1.27	-	40.82±0.63	6.33	1
	174.5±2.80	0.362±0.044	-24.4±0.66	-	52.10±0.87	6.23	2
	200.9±3.48	0.501±0.053	-21.6±2.32	94.59±0.36	55.63±0.41	6.13	3
	442.1±23.36	0.631±0.045	-23.8±0.67	89.02±0.25	69.55±0.27	5.90	6
5	321.3±3.91	0.852±0.057	-48.6±1, 00	100±0.76	75.46±0.43	6.50	0
	209.1±5.54	0.552±0.007	-38.5±1.86	-	74.24±0.45	5.86	1
	228.9±3.84	0.458±0.013	-23.4±3.32	-	84.14±0.66	5.50	2
	271.8±8.11	0.380±0.061	-25.9±2.97	93.69±0.70	90.78±0.03	5.30	3
	1210±62.18	0.917±0.074	-20.3±2.65	91.70±0.21	91.53±0.11	4.68	6
6	406.5±65.76	0.836±0.154	-43.1±2.38	100±0.14	80.43±0.79	6.50	0
	346±42.59	0.704±0.079	-33.1±1.31	-	87.10±0.46	6.14	1
	330.5±4.50	0.425±0.043	-17.5±1.06	-	90.4±0.55	5.49	2
	361±7.96	0.456±0.061	-14.8±2.29	93.86±0.33	93.86±0.17	5.29	3
	875.3±99.70	0.721±0.017	-16.0±0.35	89.34±0.34	92.02±0.08	5.01	6
7	-	-	-	100±0.37	-	-	0
	-	-	-	95.14±0.45	-	-	1
	-	-	-	94.68±0.34	-	-	2
	-	-	-	93.13±0.17	-	-	3
	-	-	-	89.29±0.26	-	-	6

[a]n = 3;SD: standard deviation between the three assays.

An increase in the mean size was more noticeable with formula 2 in comparison with both formulas 3 and 4. This may be due to formula 2 showed the highest decrease in zeta potential with time. On the other hand, the increase in the mean size may be attributed to phase transition temperature of phospholipid (T_m), that was below 0 °C, 50-60 °C and 23 °C in formulas 2, 3 and 4, respectively. At temperature above T_m, the phospholipid was in the mesomorphic state and by decreasing the repulsion force between particles, the probability of fusion may increase. Formula 3 showed the lowest increase in mean size that may be due to HSPC was in the crystalline state at storage temperature (40 °C). Adding the cholesterol by different ratios in formulas 5 and 6 did not show a positive effect on the fusion of particles, which became more noticeable after three months. In addition, the size homogenizing using ultrasonic became more difficult.

EE%

It is shown in table (8) EE% were high and related in formulas 1, 2, 3, 5 and 6, but it was obviously lower in formula 4. Adding sodium cholesteryl sulfate to formula 2 showed a little decrease in the EE% in comparison with F1. F1, F2 and F4 showed a decrease in the EE% after one month and then increased gradually with time. This may be attributed to T_m of phospholipids, which were in the mesomorphic state at storage temperature (40 °C). EE% in F3 increased gradually and did not show any decrease after one month. The lipophilicity of active substance may increase its tendency to encapsulate inside the phospholipid bilayer membrane, so encapsulation efficiency increased with time. Cholesterol can stabilize the membranes of liposomes against temperature changes, leading to lower permeability at elevated temperatures, so F5 and F6 did not show any decrease in the EE% after one month of storage in the accelerated conditions.

The total percentage

All formulas showed the relatively large decrease in the amount of the active substance. It may be due to its sensitivity to heat. Both F2 and F3 showed a little improvemet in the stability of active substance in comparison with F4 and F7. This may be attributed to the lowest EE% in F4. Both F2 and F5 showed a little improvement in the stability of active substance in comparison with F6 and F7. This may be attributed to decrease the ratio of phospholipid very much in the F6. It can be concluded that negative charge inducing SCS showed a positive effect on the stability of liposomes, HSPC showed the best results on the stability of liposomes and active substance with time, the cholesterol prevented the leakage of active substance from liposomes, whereas it did not show a positive effect on the fusion of particles.

CONCLUSION

The present study investigated the effect of several formulation variables on tretinoin loaded liposomes properties (mean diameter, zeta potential and drug encapsulation efficiency). 100 mg of soybean phosphatidylcholine, glycerol 3%v/v, adding the active substance at 30 °C, rotating speed 1300 rpm, sonication time 15 min, pH= 6.5 and ionic strength 0.025 M showed the best results.

pH= 6.5 showed the lowest decrease in pH with time and the highest EE%. Substantial decrease in both zeta potential and EE% was noticed by increasing the ionic strength. Accelerated storage conditions were applied to six formulas prepared using the previously mentioned conditions that showed the best EE% and it was thought that they are going to show the best stability with time. The effect of sodium cholesteryl sulfate, types of phospholipid and ratios of cholesterol on the stability of liposomes and active substance with time was investigated. Obvious improvement in the stability of liposomes was observed when SCS was added to the formula. HSPC showed the best results in terms of both, the stability of liposomes and active substance. Both ratios of the cholesterol prevented the leakage of the active substance from liposomes, whereas, it did not show positive effect on the fusion of particles. Further photo-stability study had been performed on final liposome formulations in comparison with the topical ethanolic solution.

The optimal formula is going to be prepared using heating method and the former mentioned conditions, based on the results of the accelerated and photo-stability study. Accelerated and long term stability study and pharmacokinetic study both (in-vitro, in-vivo drug release) are going to apply to the optimal formula in comparison with the topical ethanolic solution.

ACKNOWLEDGEMENT

The authors are grateful to University of Aleppo (Syria) for its financial support.

CONFLICT OF INTERESTS

Declared None

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