DEVELOPMENT AND OPTIMIZATION OF VITAMIN E ACETATE LOADED LIPOSOMES

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Summary

The present study describes formulation of vitamin E acetate (VEA) loaded liposomes comprising Phospholipon$^{(R)}$ 80N, cholesterol and stearic acid by ethanol injection method. The liposomal formulations were characterized for size and surface morphology, zeta potential measurements, % entrapment efficiency, rheo-logical studies,

drug disposition studies on excised skin of albino rats using Franz diffusion and cell antioxidant studies. The formulations were optimized applying 3^2 factorial design. Liposomal dispersion prepared by this method was found to increase the skin deposition of vitamin E acetate as compared to drug dispersion, conventional marketed preparation. Thus the prepared liposomes have potential application in cosmetics as well as in transdermal, mucosal, parenteral, etc. delivery systems.

Keywords: Vesicular carriers, Drug disposition studies, Factorial design.

Introduction

All living organisms suffer from the tissue damage due to regular and excessive exposure to UV light. This results in formation of different reactive oxygen species, which affects the appearance of skin. In the field of dermatology, the use of antioxidants in topical preparations is widely spread in last two decades. Recently vitamins like vitamin A, C and E are widely used as antioxidants in topical preparations.

With the help of new delivery systems known as novel drug delivery systems (NDDS) both old and new molecules can be delivered to the site in demand in a defined manner. This targeted delivery enables the molecules to produce the desired effect without disturbing the delicate bioenvironment. Among many available colloidal drug delivery systems, a class based on phospholipids has fetched much more attention than other systems because of their many meritorious features. These vesicular systems have displayed their potential to a great extent in delivering the various drugs to the target site.

Liposomes, niosomes, aquasomes, transfersomes, ethosomes are some to mention. The liposomes have emerged as most practically useful carriers for in-vivo drug delivery as majority of reports has concentrated on the use of phospholipid vesicles or liposomes as potential drug carrier systems.

Liposomes are microscopic (unilamellar or multilamellar) vesicles that are formed as a result of self-assembly of phospholipids in an aqueous media resulting in closed bilayered structures which are under extensive investigation as drug carriers for improving the delivery of therapeutic agents (1-4). Small-sized liposome's have several advantages over larger liposome's in drug delivery systems, and the ethanol injection method is one of the suitable technique to obtain spontaneous formation of liposome's having small average radius. A narrow distribution of small liposome's (less than 100 nm) can be obtained by simply injecting an ethanolic lipid solution in an aqueous media in one step, without the use of extrusion in the literature and following the original paper by Batzri and Korn (5). Up till now few reports on the injection method for different application have been appeared (6-9). However this method is also amenable for industrial application (10, 11). Choice of solvent is usually ethanol, but other alcohol such as isopropanol, butanol, etc. are also used (12-14). The present study was undertaken to overcome the problem of barrier effect of the skin, to protect the actives from photodegradation, to improve the delivery of actives (Vitamin E Acetate**)** across the skin.

Methods

Preparation and Optimization of Liposomes

Liposomes were prepared by modified ethanol injection method as described by Batzri and Korn (5). The required amount of VEA (12mg), (Phospholipon$^{(R)}$ 80N (P80N), cholesterol (CH) and stearic acid (SA) were dissolved in 1 ml of ethanol. Ethanolic solution was rapidly injected into 10 ml of double distilled water (DDW) under stirring at 500 rpm for 15 min using Teflon coated magnetic bead.

The 3^2 factorial design approach was utilized in formulating different batches in order to study the effect of variables on liposome performance and characteristics. Amount of P 80N (Variable X1) and CH (Variable X2) were selected as two independent variables. Vesicle size, encapsulation efficiency (EE) and drug deposition in the rat skin were selected as dependent variables. Amount of SA (3 mg) and VEA (12 mg) were kept constant. Values of all variables and batch codes are as shown in Table1.

Table1 Composition of liposomal formulations as per factorial design

Formulation Code	Variable X_1	Variable X_2
LP-1	60 (+1)	12(+1)
LP-2	60(+1)	09(0)
LP-3	60(+1)	06(-1)
LP-4	50(0)	12(+1)
LP-5	50(0)	09(0)
LP-6	50(0)	06(-1)
LP-7	40(-1)	12(+1)
LP-8	40(-1)	09(0)
LP-9	40(-1)	06(-1)

*Values in parentheses indicates coded levels

Characterization of Liposomes

Size Distribution

Mean vesicle size and size distribution of empty and drug-loaded liposomes was determined using Zetasizer 300HSA (Malvern Instruments, Malvern, UK) based on photon correlation spectroscopy. Analysis (n = 3) was carried out for 100 at room temperature by keeping angle of detection at 90^{o}.

Zeta Potential (ζ) Determination

Charge on empty and drug loaded vesicles surfaces was determined using Zetasizer 300HSA (Malvern Instruments, Malvern, UK). Analysis time was kept for 60 s and average zeta potential and charge on the liposomes was determined.

Drug Content

The liposomes were ruptured using sufficient volume of methanol and it was then subjected to HPLC analysis. Estimation of VEA was carried out using HPLC (JASCO, Japan) equipped with a UV detector; Detection was carried out at 280 nm. Column used was C-18 (0.5 µm, Hyersil$^{(R),}$ 25 mm x 4.6 mm, Thermo) attached with guard column (ODS, Hypersil $^{(R),}$ 20 mm x 4 mm, Thermo). Drug was eluted using methanol as mobile phase at a flow rate of 1.6ml/min.

Encapsulation Efficiency (EE)

EE was determined by minicolumn centrifugation method as described by Fry etal (15). Sephadex$^{(R)}$ G25 M (10% w/v) was added in DDW and was kept aside for 24 h for swelling. To prepare minicolumns, Whatman filter pad was inserted in 1 ml syringe and swelled sephadex was added slowly to it. Care was taken to avoid air entrapment in the column. Excess amount of water was removed by spinning the column at 2000 x g for 3 min using Eppendorff Centrifuge 5810 R (Hamburg, Germany), Liposome dispersion (100 µl) was slowly added on the prepared

column and centrifuged as earlier. Procedure was repeated by adding eluted liposomes on the fresh column. Obtained eluted liposomes were ruptured using sufficient volume of methanol and percent encapsulation was calculated from total amount of VEA present in 100µl of liposomes by HPLC using equation1. Method was validated by applying free drug solution instead of liposomes[32].

$$\%EE = \frac{Qe}{Qt} X100 \quad \text{……..Eq.1}$$

where Q_e – Amount of encapsulated VEA,
Q_t – Total amount of VEA

Drug Deposition Studies

Deposition study was performed on the excised skin of sacrificed swiss albino rats weighing 225-250 g using Franz Diffusion cell. The study was in compliance with guidelines of institutional animal ethical committee (IAEC). Abdominal skin of rat was shaved and skin was carefully separated. Subcutaneous fat was carefully removed using a scalpel. Skin section thus obtained was mounted on Franz Diffusion cell having surface area of 3.91 cm^2 and receptor compartment having a capacity of 22 ml. The epidermal side of the skin was exposed to ambient condition while dermal side was kept facing to receptor solution. Receptor compartment was filled with DDW as diffusion medium maintained at $37^oC \pm 0.5^oC$. Tween 80 (0.5% v/v) was added to the reservoir solution to maintain the sink condition and was kept under stirring at 500 rpm. Diffusion cell was protected from the light. Skin was saturated with diffusion medium for 1 h prior to application of sample. A dose equivalent to 30 IU of VEA encapsulated in liposomes was applied on donor compartment and diffusion was carried out for 24 h. The cell was dismantled after 24 h and skin was carefully removed. Drug present on the surface of the skin was removed by using method described by du Plessis et al (16), using Scotch Tape (Scotch Magic Tape, 810, Birla 3M Ltd. Banglore, India). After stripping, the skin was cut into small pieces and drug present in the skin was extracted in methanol under bath sonication for 2 h and the extract was

estimated by HPLC. Similarly drug deposition from VEA dispersion in water (control preparation) was carried out to find out whether the entrapment of VEA in liposomes improves the skin drug deposition. Analysis of data was carried out using 'PCP Disso V 3' software (HPC, PCP, Pune, India).

Statistical analysis

Liposomes were prepared using ethanol injection method and Obtained data were subjected to multiple regression analysis using Unistat Software (Megalon, USA). The insignificant variables were removed, and adequacy of fitted model was checked by analysis of variance (ANOVA).The data were fitted in equation 2.

$$Y = \beta_0 + \beta_1 X1 + \beta_2 X_2 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_1 2 X_1 X_2 \quad \text{.Eq 2}$$

Stability Study

The liposome dispersion (LP-6) was filled in amber colored bottle and aluminium collapsible tube. The samples were kept at 30^o C/65% RH for three months. Effects of temperature and relative humidity (RH) on the vesicle size, content uniformity, %EE and drug deposition in rat skin were monitored at 0, 30, and 90 days for liposomal dispersion.

Results and Discussion

Preparation and Optimization of Liposomes

Liposomes were prepared using ethanol injection method and optimization was performed by 3^2 Factorial design to assess the influence of various formulation variables upon the features of liposomes (Table2).

Effect of Variables on Encapsulation Efficiency (EE)

Determination of EE is an important parameter in case of liposomes as it may affect the drug release and skin deposition. In the present study the observed EE for all

batches were in the range of 98-101%. When regression was applied to EE it was observed that it was insignificantly affected by the applied procession variables. High EE observed for VEA may be attributed to the highly lipophilic nature of the drug. The lipophilic nature of VEA allows for its spontaneous intercalation with the lipids thus promoting entrapment in the vesicles.

Effect on Size and Size Distribution

The number of reports revealed that the size and size distribution of the liposome determines their in-vivo or ex-vivo performance. The ethanol injection method was found to produce polydispersity index of less than 0.3 which indicates obtained liposome population have narrow size of distribution. It was observed that the relative amount of P 80N CH and VEA was found to play an important role in the determining of vesicles size. Size of the empty vesicles was found to be in the range 124-206 nm.

To understand the effect of lipid concentration on vesicle size, coefficient observed for the both empty and drug loaded liposomes size was fitted in equation 2 to generate equation 3 and 4, respectively. The effects are more clearly understood from the response surface plots, which were generated for both the parameters. After the application of regression analysis following equations were generated.

$$Y = 174.34 + 29.217X_1 + 14.708X_2 - 9.786X_1X_1 + 6.342X_2X_2 \quad \text{Eq.3}$$

$$Y = 161.044 + 37.716X_1 + 16.142X_2 \quad \text{Eq.4}$$

A positive correlation was observed for both he variables X_1 (P 80N) and X_2 (CH) in case of vesicle size of empty liposomes (eq.3) $r^2 = 0.9716$) as well as drug loaded liposomes (eq.4; $r^2 = 0.9567$). This with increase in the concentration of P 80N and CH vesicle size was found to be increased in empty and drug loaded before (Figure1 and 2). When we compared the coefficient value of both the variables we observed that the effect of P 80 N was more prominent than the effect of CH.

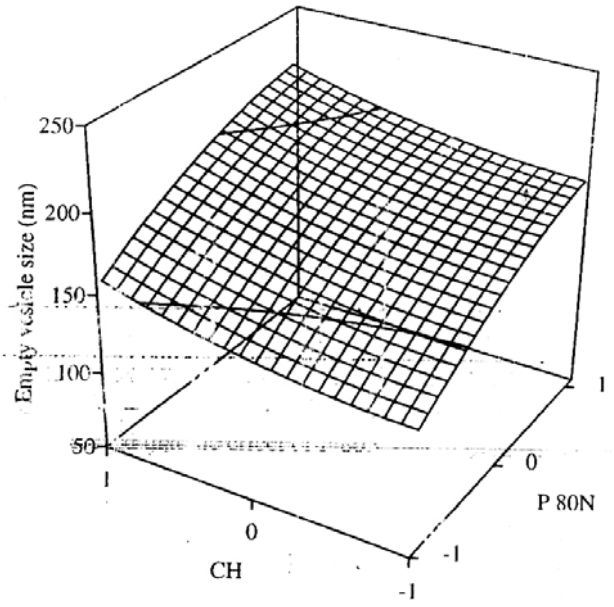

Figure1Effect of lipid concentration on vesicle size of empty liposome

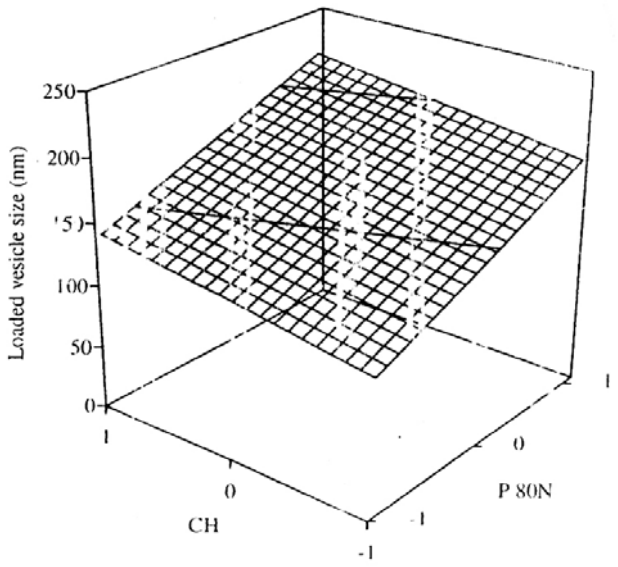

Figure2 Effect of lipid concentration on vesicle size of drug loaded liposomes

Table 4.2: Characterization of Liposomal Formulations

Batch code	Vesicle size of empty liposomes (nm ±SD)	Vesicle size of drug loaded liposomes (nm ±SD)	Encapsulation efficiency (% EE ±SD)	Polydispersity index(PI) of drug loaded liposomes	ζ of empty liposomes (mV) ±SD	ζof drug loaded liposomes (mV) ±SD
LP-1	206.50 ±1.25	208.22 ± 1.58	100.45± 3.44	0.280	-52.00 ± 0.99	-53.87 ± 0.51
LP-2	198.25 ± 1.53	199.45 ± 1.67	99.65 ± 0.76	0.265	-50.13 ± 1.10	-52.93 ± 0.85
LP-3	189.15 ± 1.51	192.13 ± 1.86	98.68 ± 0.76	0.233	-47.00 ± 1.13	-48.70 ± 0.62
LP-4	201.64 ± 1.48	189.00 ± 1.48	98.37± 2.09	0.189	-42.50 ± 0.72	-50.27 ± 0.42
LP-5	169.15 ± 1.33	161.75 ± 1.38	98.66 ± 1.93	0.204	-30.67 ± 4.81	-51.07 ± 1.57
LP-6	164.95 ± 1.44	136.00 ± 1.33	100.95± 3.25	0.211	-27.37 ± 1.46	-35.33 2.71
LP-7	158.40 ± 1.62	142.15 ± 1.31	99.78 ± 2.11	0.198	-26.90 ± 2.71	-29.30 ± 1.87
LP-8	136.05 ± 1.38	123.90 ± 1.66	98.71 ± 4.94	0.201	-30.67 ± 3.72	-33.57 ± 1.16
LP-9	124.25 ± 1.23	116.55 ± 1.33	100.55± 3.82	0.200	-39.67 ± 0.74	-45.33 ± 1.00

Determination of Zeta (ζ) Potential

A ζ value > ± 30 mV is essential for effective stability and to inhibits aggregation (17). In the present study the potential obtained for empty and drug loaded liposomes are shown in Table 2. It was observed that ζ potential of vesicles was increased after the addition of drug to liposomes. The values of potential (-27 to –52 mV and –29 to –54 mv for empty and loaded vesicles, respectively) showed prepared liposome have sufficient charge to inhibit aggregation of vesicles.

Effect on Drug Deposition in the Skin

There are several methods reported for the performance of drug deposition in the skin which includes diffusion, microdialysis, microscopic studies, etc. (18,19). The present investigation deals with drug deposition determined by diffusion method, which is most widely used method. Drug deposition in the skin for different batches is depicted in figure3. To check the performance of VEA encapsulated in liposomes, plain drug dispersion prepared in water was used as a control preparation, and it was observed that only 6.91± 0.31 μg/cm^2 of VEA was found to be deposited in the skin. The encapsulation of VEA in liposomes showed more than two times increase in deposition in the skin. The amounts of P 80N and CH were found to be key variables affecting the drug deposition. Comparatively less amount of VEA was deposited from batches LP-1 to LP-3. This may be due to comparatively higher particle size and high level of P 80 N (+1).
Among all batches, LP-6 which had intermediate vesicle size (164.95 ±1.44 nm) showed maximum drug deposition of 13.31± 1.66 μg/cm^2.

Thus to better understand the effect of both the variables on drug deposition response surface plots were generated using equation 5.

$Y = 7.443 – 1.893X_1 – 2.796X_2$ ---------- Eq.5

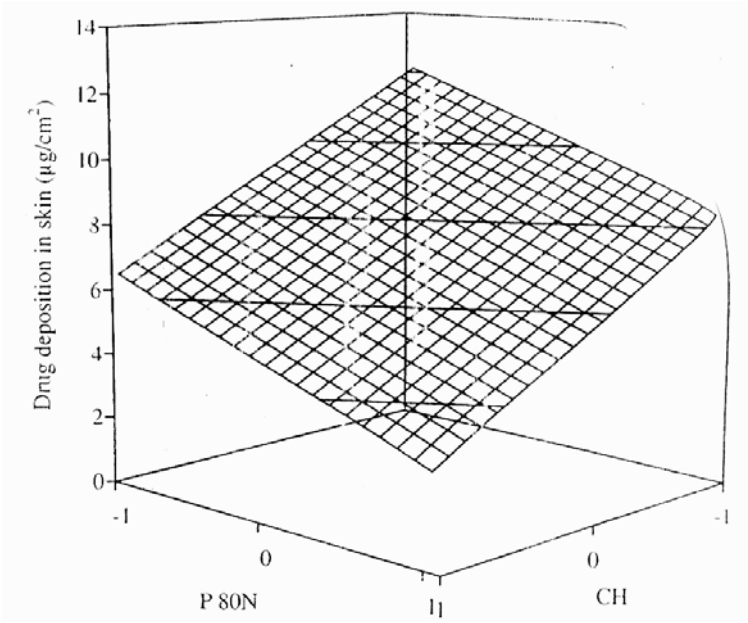

Figure 3 Effect of lipid concentration of VEA deposition

Drug deposition was found to be an inverse function of X1 (P80N) and X2(CH). Drug deposition increased with decreasing amount of P 80N and amount CH (Figure3).
A good degree of fitness was obtained for this parameter. But discrepancy in the obtained size and drug loaded vesicle size could be due to introduction of some additional bio-variables, which varies from skin to skin and are beyond precise control resulting in scattering of data.

Stability Study

Stability of liposome dispersion (LP-6) was carried out for three months at 30 °C/65% RH. Insignificant (P>0.001) effect was observed on EE and content uniformity throughout the stability period for liposome dispersion. Similarly insignificant (p>0.001) increase in vesicle size was observed (Table4) with time, which might be attributed to very slight fusion of the liposomes. This increase in vesicle size might be due to presence of surface negative charge and high zeta potential on liposomes, which either avoids or

delays formation of liposome aggregates due to electrostatic repulsion. Deposition of VEA in the skin from liposomal dispersion was found to insignificantly decreased ($p>0.001$) during stability period (Table4) indicating prepared liposomes are stable for three months.

Table 4 Effect on vesicle size and skin drug deposition during stability (n=3)

Parameters	LP-6		
	Initial	30 days	90 days
Vesicle Size (nm±SD)	132.4±0.21	144.5±0.48	151.2±1.1
Drug Deposition (ug/cm^2±SD)	13.31±1.66	12.38±2.58	12.30±1.91

Conclusion

Preparation of liposomes using3^2 factorial design was found to be well-suited and sound approach to obtain stable liposomal formulations of vitamin E acetate. Loading of the highly lipophilic drug such as vitamin E acetate in the liposomes greatly influences the micromeritics of the vesicles. Different variables such as amount of phospholipid, amount of stabilizer and lipid: drug ration have a profound effect on the vesicle size and drug deposition in the rat skin. Increased drug deposition in rat skin as compared to control drug dispersion and conventional marketed cream suggest that liposomal formulation promotes drug deposition in the rat skin and hence has potential for dermal delivery. Liposomal dispersion were found to be stable for three months at 30°C and 65 % RH.

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