Original Article

# Development and characterization of hyaluronic acid-loaded ethosomes for topical application

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## ABSTRACT

**Background:** Skin aging is such a problem in which actives should be delivered in a deeper layer of skin. Hyaluronic acid (hyaluronan) is a major component of skin, where it is involved in tissue repair. It is also important in maintaining skin firmness and structure. The topical application of hyaluronan helps in maintaining healthy collagen levels and moisturizes the skin. Hyaluronic acid does not easily penetrate the skin when it is applied topically. **Methods:** Ethosomal formulations were prepared by the "Hot Method." Optimization of ethosomes was done to get optimal vesicle size. The size, size distribution, and zeta potential of optimized carriers were determined by Zetasizer. An *in vitro* drug release study of ethosomal formulation was performed phosphate buffer pH 6.8. **Results:** The ethosomal formulation was found to be optimal for topical use. It was also observed that sodium hyaluronate released from the ethosomes was characterized by a slow release of 25.52 ± 2.08% for 4 h and followed by controlled release. Stability studies indicated that sodium hyaluronate loaded ethosomes are found to be more stable at refrigerated conditions rather than room temperature. **Conclusion:** Thus, this study described a method for the preparation of ethosomes with lecithin, ethanol, and water, which could be utilized for topical drug delivery successfully.

**Keywords:** Ethosomes, hyaluronic acid, skin aging, topical delivery

## INTRODUCTION

Topical delivery for the treatment of skin disorders offers numerous potential advantages over systemic therapies, such as those involving the use of oral or parenteral products. These include avoidance of hepatic first-pass metabolism, improved patient compliance, and ease of access to the absorbing membrane, that is, the skin. Besides, by directly administering the drug to the pathological site, any adverse effects associated with systemic toxicity can be minimized.[1]

However, the targeted delivery of drugs for the treatment of topical disorders is not trivial. The physiological function of the stratum corneum, the outermost and non-viable layer of the skin, is to act as a protective barrier for the body and as such, it is particularly effective at preventing the permeation of hydrophilic molecules including some drugs into deeper skin layers, where viable cells are located.[2]

Skin aging is such a problem in which actives should be delivered in a deeper layer of skin.[3,4] Hyaluronic acid (hyaluronan) is a major component of skin, where it is involved in tissue repair. It is also important in maintaining skin firmness and structure. Photo damaged skin produces less hyaluronan and contributes to hyaluronan deficiency, thus resulting in wrinkles.[5,6]

The topical application of hyaluronan helps in maintaining healthy collagen levels and moisturizes the skin. Sodium hyaluronate is a powerful humectant that attracts and holds on to water, making it the ultimate skin moisturizer. This helps to hydrate the skin and keep it moist and supple. Since it attracts and binds to water, it causes slight swelling. This swelling helps to reduce the appearance of fine lines and wrinkles and gives skin a more youthful appearance. Furthermore, the subtle swelling increases volume, which makes the skin look younger.[3]

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Hyaluronic acid does not easily penetrate the skin when it is applied topically. Additional methods of chemical or physical penetration enhancement available to traverse this barrier are associated with potential side effects as these promote the transport of drugs through the skin into the bloodstream.[3,7]

Consequently, in topically applied formulations, there is a need for an ultra-flexible vesicular carrier system like ethosomes of the optimum size that helps the drug to negotiate successfully the stratum corneum but then avoids further penetration across the deeper layers of the skin (hypodermis) and into the bloodstream.[4]Thus, in present work, hyaluronic acid-loaded ethosomes were developed and evaluated for dermatological benefits.

## MATERIALS AND METHODS

### Materials

Sodium hyaluronate was obtained from Kartik Enterprises, Mumbai. Soya phosphatidylcholine (lecithin) was procured from Hi-Media Laboratory Pvt. Ltd., Mumbai. Propylene glycol, ethanol potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, and n-propanol were purchased from Central Drug House, New Delhi. All other reagents used in the present investigation were of analytical grade.

### Formulation of sodium hyaluronate loaded ethosomes

Ethosomal formulations were prepared by the "Hot Method.[4]" The drug concentration was fixed as 0.05% w/w. The varying amount of soy lecithin (phospholipid) and ethanol was used. Accurately weighed quantity of the drug was dissolved in ethanol and propylene glycol. The soy lecithin was dissolved in water at 40°C in another beaker. The drug solution was then added slowly to soy lecithin dispersion of water at 40°C with 1700 rpm in a closed vessel and was stirred for 30 min. The final preparation was subjected to ultra-sonication for an hour with a cycle of 10 min. The evenly dispersed ethosomal vesicles were formed.

### Optimization of ethosomes

Optimization of ethosomes was done using the traditional method of optimization, that is, one variable at a time. The following independent variables (formulations and process variables) were selected for the optimization:

- Amount of lecithin
- Amount of ethanol.

Vesicle size and encapsulation efficiency were considered as dependent variables

#### *Optimization of amount of lecithin*

First, the amount of lecithin was optimized, keeping drug concentration, ethanol quantity, and other parameters constant. The lecithin was optimized to get the maximum entrapment efficiency (EE%) and desired vesicle size. Different batches were formulated, as shown in Table 1.

#### *Optimization of the amount of alcohol*

After selecting the optimum lecithin amount, the amount of drug, propylene glycol, lecithin (optimized amount), and other parameters was kept constant and the effect of the amount of ethanol was optimized. Different batches were formulated, as shown in Table 2.

### Characterization of ethosomes

#### *Vesicle size and size distribution analysis*

The size, size distribution, and zeta potential of optimized carriers were determined by Zetasizer (Zetasizer Nano ZS+MPT-2 Autotitrator, Malvern Instruments Ltd., UK.). Dispersions were dispersed in an appropriate medium (i.e., distilled water) and put into the cuvettes of Zetasizer and the size of the carrier systems and polydispersity index (PDI) was measured.[8,9]

#### *EE%*

The drug was estimated in ethosomes by the ultracentrifugation method. The total volume of ethosomes suspension was measured and 2 mL of this formulation was transferred to a 10 mL centrifuge tube. The suspension was diluted with distilled water up to 5 mL and centrifuged at 2000 rpm for 20 min to separate unentrapped drug in the formulation. Ethosomes were separated by ultracentrifugation at 20,000 rpm for 30 min.[4,10] Supernatant and sediment were recovered and their volume was measured. Sediment was diluted up to 5 mL with n-propanol to lyse the ethosomes. The unentrapped and entrapped drug contents were analyzed by estimating drug in supernatant and ethosomes (sediment) spectrophotometrically.

$$\%EE = \frac{\text{Amount of drug entrapped}}{\text{Amount of drug added}} \times 100$$

### *In vitro* drug release studies

#### *Activation of dialysis membrane*

The dialysis membrane was exposed to running water for 12 h to remove glycerin-based contents. The sulfur-based contents were removed by treating the membrane with 0.03% (w/v) sodium sulfite at 70°C for 20 min. Then, it was washed with hot water at 70°C for 2 min followed by exposure to 0.2% w/v solution of sulfuric acid

**Table 1: Optimization of lecithin**

| Formulation code | Lecithin (mg) | Ethanol (g) | Encapsulation efficiency (%) | Vesicle size (nm) |
|---|---|---|---|---|
| ET-1 | 100 | 3 | 38.53±2.3 | 179.1±4.2 |
| ET-2 | 200 | 3 | 47.63±1.47 | 188.6±6.7 |
| ET-3 | 250 | 3 | 53.42±2.38 | 197.5±3.8 |
| ET-4 | 300 | 3 | 72.56±2.34 | 275.4±2.9 |
| ET-5 | 350 | 3 | 68.72±1.21 | 250.2±5.7 |

*All values are expressed as mean±SD, n=3

**Table 2: Optimization of alcohol content**

| Formulation code | Lecithin (mg) | Ethanol (g) | Encapsulation efficiency (%) | Vesicle size (nm) |
|---|---|---|---|---|
| ET-6 | 300 | 2 | 67.74±1.13 | 284.4±5.1 |
| ET-4 | 300 | 3 | 72.56±2.34 | 275.4±2.9 |
| ET-7 | 300 | 3.5 | 65.74±1.24 | 267.7±4.6 |

*All values are expressed as mean±SD, n=3

for 5 min.[11,12] This acidification was followed by treatment with hot water to remove excess acid. The treated membrane was kept in alcohol until used for *in vitro* drug release studies.

### *In vitro drug release*

An *in vitro* drug release study of ethosomal formulation was performed phosphate buffer pH 6.8. The ethosomal formulation (2 mL) was separately placed in dialysis bags, tied at both ends, and was suspended in a beaker containing 20 mL of buffer maintained at 37°C under mild agitation on the magnetic stirrer.[1] At pre-determined time intervals, aliquots (1 mL) were withdrawn from the receptor compartment and after each withdrawal of the sample, the receptor compartment was replenished by the same volume of fresh media to maintain a constant volume throughout the study. The samples were then analyzed for absorbance by (ultraviolet) UV spectrophotometer at $\lambda_{max}$ 325 nm.

## Storage stability studies

Storage stability is an important consideration in the development of the pharmaceutically acceptable product. In the present study, the developed formulation was stored in a refrigerator (5 ± 3°C) and at room temperature (25 ± 2°C)/RH 60 ± 5% for 3 months. During this time, at periodic intervals, the vesicular formulation was evaluated for any change in the vesicle size and residual drug content.[4]

# RESULTS AND DISCUSSION

## Formulation and optimization of sodium hyaluronate loaded ethosomes

Ethosomal formulations were prepared by the "Hot Method."

Optimization of ethosomes was done to get optimal vesicle size. The following formulations and process variables were optimized:

- Amount of lecithin
- Amount of ethanol.

First, the amount of lecithin was optimized, keeping drug and ethanol quantity constant. The lecithin was optimized to get the maximum EE%, desired vesicle size. It was observed that, on increasing lecithin content, the vesicle size also increased. However, the EE% was first increased up to a level, and then it is decreased. This might be due to a lesser proportion of ethanol in comparison to the quantity of lecithin. The results are shown in Table 1 and Figure 1.

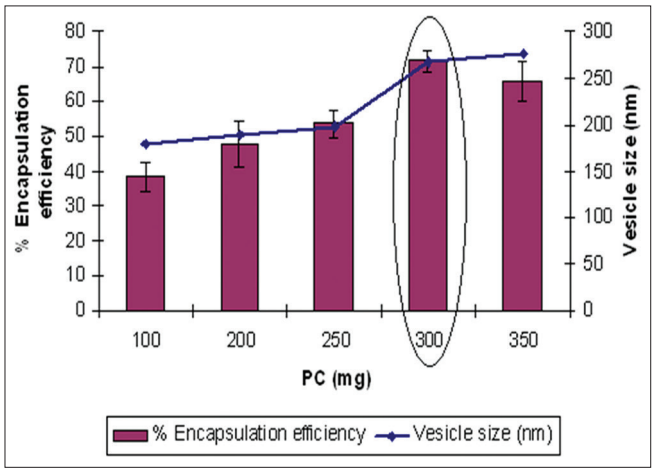


**Figure 1:** Optimization of lecithin quantity

After selecting the optimum lecithin amount, the amount of drug, propylene glycol, and lecithin (optimized amount) was kept

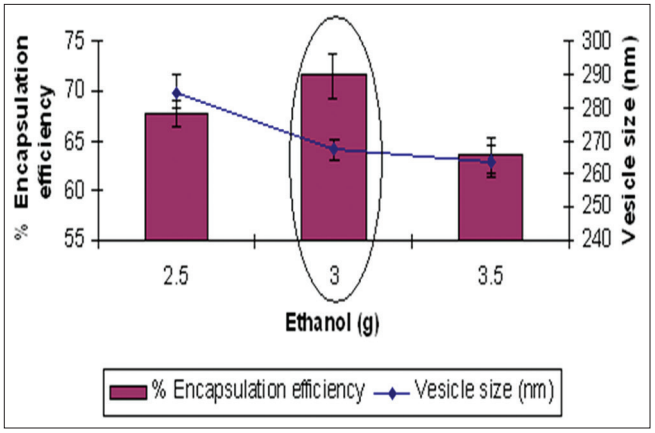


**Figure 2:** Optimization of ethanol quantity

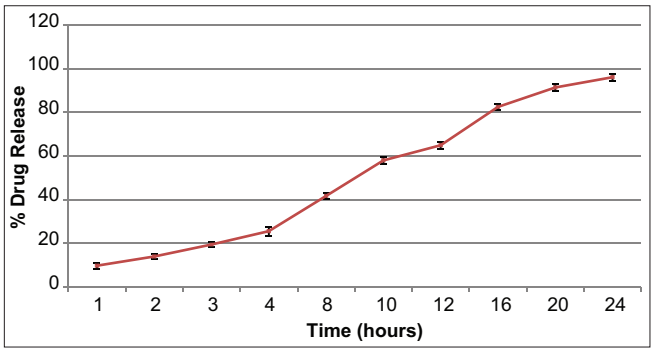


**Figure 3:** *In vitro* drug release profile from ethosomes

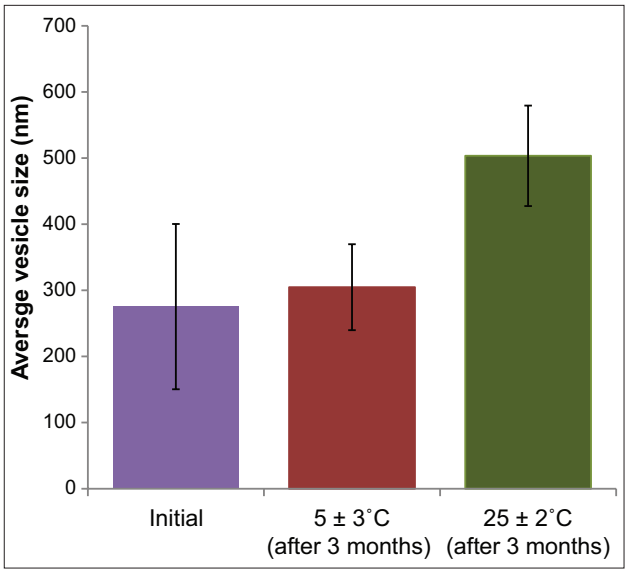


**Figure 4:** Effect of storage on average vesicle size of ethosomal formulation

constant and the effect of the amount of ethanol was optimized. As the ethanol concentration increased from 2 g to 3 g, there was an increase in the EE% and with further increase in the ethanol, the vesicle membrane becomes more permeable which leads to a decrease in the EE%. The results are shown in Table 2 and Figure 2. The size distribution of ethosomes ranged between about 179 nm and 284 nm and is known to be influenced by the composition of ethosomes. The size of the vesicles decreased when the ethanol concentration was increased. The largest vesicles of 284.4 ± 5.1 nm were present in the preparation containing minimum ethanol. This difference in the size of ethosomal formulations is because of the presence of different concentrations of ethanol. Probably, ethanol causes a modification in a net charge of the system and confers it some degree of steric stabilization that may lead to a decrease in mean vesicle size. As a vesicle structured neotype of drug carrier, ethosome has the characteristics of good deformability, high EE%, good permeability, and good stability, which enable them to effectively carry the drug through the skin and to penetrate through cuticle to get into deeper layers. It was the good fluidity and deformability given by ethanol that enabled ethosomes to carry the drug to penetrate through the eyelet, 1/5–1/10 less than its size without much change in shape under the pressure of hydration, to penetrate through the cell membrane and release drug more effectively than liposomes.

## Characterization of ethosomes

### *Vesicle size and size distribution analysis*

The size, size distribution, and zeta potential of optimized carriers were determined by Zetasizer; results of the optimized batch are shown in Table 3.

### *EE%*

The EE% was estimated by the ultracentrifugation method and results for the optimized formulation are shown in Table 3.

### *In vitro drug release studies*

An *in vitro* drug release study of ethosomal formulation was performed phosphate buffer pH 6.8. The results are shown in Table 4 and Figure 3. It was observed that sodium hyaluronate released from the ethosomes was characterized by a slow release of 25.52 ± 2.08% for 4 h and

**Table 3: Optimized parameters of the final formulation**

| Parameters | Optimized ethosomes |
|---|---|
| Vesicle size (nm) | 275.4±2.9 nm |
| PDI | 0.12±0.034 |
| Zeta potential | −21.23±2.87 mV |
| EE % | 72.56±2.34% |

PDI: Polydispersity index, EE: Entrapment efficiency

**Table 4: *In vitro* drug release profile from ethosomes**

| Time (h) | % Drug release from ethosomes |
|---|---|
| 1 | 9.70±1.36 |
| 2 | 13.88±1.34 |
| 3 | 19.37±1.25 |
| 4 | 25.52±2.08 |
| 8 | 41.67±1.27 |
| 10 | 57.92±1.56 |
| 12 | 64.88±1.53 |
| 16 | 82.45±1.28 |
| 20 | 91.33±1.68 |
| 24 | 96.03±1.61 |

Values are expressed as mean±S.D., n=3

**Table 5: Effect of storage on average vesicle size of the formulation**

| Formulations | Average vesicle size (nm) | | |
|---|---|---|---|
| | Initial | 4±1°C | 25±2°C (RH 60±5%) |
| | | After 3 months | After 3 months |
| Optimized ethosomes | 275.4±125.87 | 304.76±65.32 | 503.45±76.81 |

Values are expressed as mean±S.D., n=3

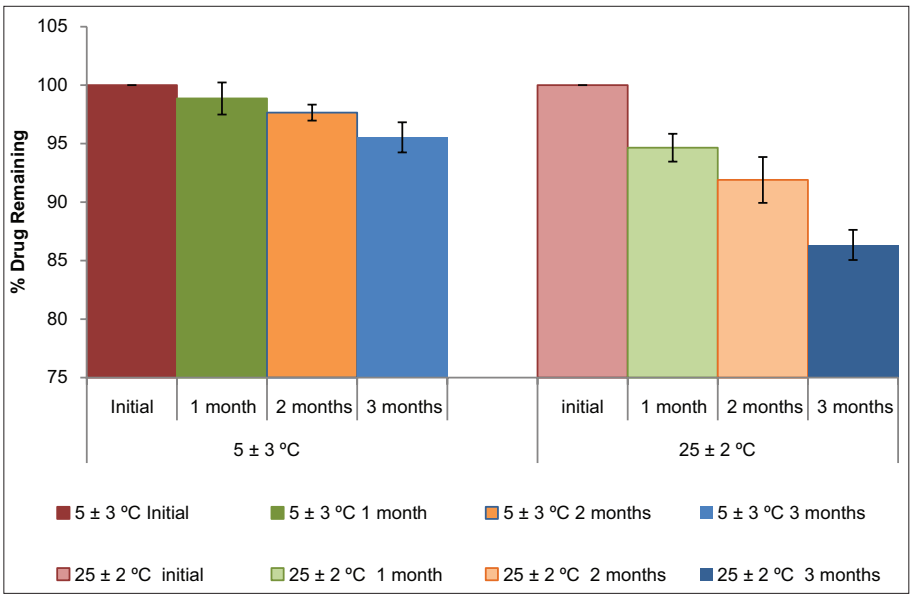


**Figure 5:** Effect of storage on residual drug content (%)

**Table 6: Effect of storage on residual drug content (%)**

| Formulation | Residual drug content (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Initial | 4±1°C | | | 25±2°C (RH 60±5%) | | |
| | | 1 month | 2 months | 3 months | 1 month | 2 months | 3 months |
| Optimized ethosomes | 100 | 98.86±1.37 | 97.65±0.68 | 95.54±1.29 | 94.65±1.19 | 91.90±1.96 | 86.34±1.29 |

Values are expressed as mean±SD., n=3

followed by controlled release. The percentage of sodium hyaluronate release from ethosomes after 24 h was found to be 96.03 ± 1.61%.

## Storage stability studies

Although, vesicular systems are generally stored only in the refrigerated conditions, yet, it may be anticipated that the formulation with the suitably designed carrier construct(s) might impart improved stability. In the present study, the formulation was stored in the refrigerator (5 ± 3°C) and at room temperature (25 ± 2°C) for 3 months.

### *Effect of storage on average vesicle size*

The formulations were stored at different temperatures and the size of ethosomes was determined after 3 months. The results are shown in Table 5 and Figure 4. No significant change in vesicle size was observed after storage at 5 ± 3°C as vesicle size was recorded as 304.76 ± 65.32 nm. However, there was a significant increase in vesicle size (503.45 ± 76.81 nm) when stored at 25 ± 2°C. This may be due to the fusion of the bilayer membrane of vesicles at a higher temperature.

### *Effect of storage on residual drug content (%)*

Residual drug content (%) of the different formulations was determined by disrupting the ethosomes with n-propanol at various time intervals (1 month, 2 months, and 3 months). Further estimation of drug content was done by UV spectroscopy at 325 nm. The results are shown in Table 6 and Figure 5.

The results indicated that refrigerated temperature is suitable for their storage as after 3 months, the residual drug content estimated was 95.54 ± 1.29%. However, at 25 ± 2°C, the residual drug content (%) after 3 months was estimated to be 86.34 ± 1.29%. A significant change in drug content was observed at 25 ± 2°C.

The above results can be used to conclude that formulated sodium hyaluronate loaded ethosomes are found to be more stable at refrigerated conditions rather than room temperature. This may be due to the fusion of the bilayer membrane of vesicles at a higher temperature.

# CONCLUSION

This study described a method for the preparation of ethosomes with lecithin, ethanol, and water. The optimization process resulted in the development of vesicles of desired vesicle size with the highest EE%. Further pre-clinical and clinical studies need to be carried out to find out distribution behavior in the skin tissues. The clinical efficacy of a topically applied drug depends not only on pharmacological properties but also on the availability of the drug at the target site.

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