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Artículos originales

Skeletal muscle relaxant activity of different formulation of span 60 niosomes

Actividad relajante del músculo esquelético de diferentes formulaciones de niosomas span 60

Raj Kumar Keservani
Surya Prakash Gautam

1IKG Punjab Technical University, Jalandhar, Research Scholar, Punjab, India.
2CT Institute of Pharmaceutical Sciences, Shahpur Campus, Jalandhar, Punjab, India

Correspondence
Raj Kumar Keservani
rajsops@gmail.com

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Resumen

Introducción: La espasticidad es una enfermedad de las neuronas motoras que se manifiesta como aceleración del tono muscular y rigidez. Los niosomas se pueden formular con la ayuda de un ajuste adecuado de los parámetros del proceso para mejorar el atrapamiento del baclofeno y mantener la liberación del fármaco.

Método: El objetivo principal de este estudio es comparar diferentes formulaciones de niosomas span 60 que contienen baclofeno para la actividad relajante del músculo esquelético en ratones (ratones albinos suizos) mediante el método de rota rod.

Resultados: El tamaño de partícula de los niosomas formulados estaba en el rango de 3,62 ± 0,54-4,08 ± 0,64 µm y eran suaves, de ajuste circular y generalmente multilaminares pequeños. La eficiencia de atrapamiento de la formulación optimizada fue 88,44 ± 0,28%. El porcentaje de liberación acumulada de fármaco más extremo fue 87,88 ± 8,66% después de 10 h. La formulación almacenada a una temperatura de 4 ± 2 °C muestra una mejor estabilidad (96,65 ± 0,45) en comparación con la temperatura elevada. Se utilizaron ratones albinos suizos para el estudio in vivo y mostraron una acción relajante muscular mejorada hasta donde no. de caídas desde el aparato de varilla de rotación (valor de p = 0,001).

Conclusiones: No obstante, se observó que los ratones tratados con diazepam tenían una mayor relajación muscular que cualquier dosis de formulación probada. La formulación F9 muestra una mejor actividad relajante del músculo esquelético en comparación con F6, F7, F8 y F10 en ratones (ratones albinos suizos) mediante la técnica de rota rod.

Palabras clave: Niosomas; baclofeno; diazepam; ratones albinos suizos; relajante del músculo esquelético.

Abstract

Introduction: Spasticity is a disease of motor neurons that manifests as accelerated muscle tone and stiffness. The niosomes can be formulated with the aid of proper adjustment of process parameters to enhance baclofen entrapment and sustaining the drug release.

Method: The main purpose of this study is to compare different formulations of span 60 niosomes containing baclofen for skeletal muscle relaxant activity on mice (Swiss albino mice) by rota rod method.

Results: The particle size of formulated niosomes was in the range of 3.62±0.54-4.08±0.64 µm and these were smooth, circular fit and generally small multilamellar. Entrapment efficiency of optimized formulation was 88.44±0.28 %. The most extreme % cumulative drug release was 87.88±8.66% after 10 h. The formulation stored at 4±2 °C temperature shows better stability (96.65±0.45) contrasted with raised temperature. Swiss albino mice were utilized for in vivo study and displayed improved muscle relaxant action as far as no. of tumbles from rota rod apparatus (p value =0.001) are concerned.

Conclusions: Nonetheless, diazepam treated mice were observed to have higher muscle relaxation than any dose of formulation tested. The formulation F9 shows better skeletal muscle relaxant activity as compared to F6, F7, F8 and F10 on mice (Swiss albino mice) by the rota rod technique.

Keywords: Niosomes; baclofen; diazepam; Swiss albino mice; skeletal muscle relaxant.
**Highlights**

1. Topically applied drugs can be delivered as a niosomal vesicular system.
2. Vesicular system can be used to enhance the penetrability of the drug via skin.
3. There was observed an improved permeation of baclofen confined in niosomes as compared to plain drug.

**Introduction**

The drug absorption via the skin is facilitated by transdermal drug delivery system. This offers several advantages over orthodox delivery pathways like intravenous or oral for systemic and local drug delivery. This alleviates the load on patients due to intravenous drug delivery and reduces ill effects of first pass effect of the liver, providing therapeutics in controlled manner. There are a number of active (thermal ablation, iontophoresis, microneedles, ultrasound) and passive (vesicular drug carriers, chemical penetration enhancers, prodrug approaches) techniques are reported to demonstrate potential with success in several instances. Accordingly, many drugs are available in the market as novel transdermal dosage forms.

The non-ionic surfactant structures are closed bilayer vesicles in watery media dependent on amphiphilic nature of the surfactant utilizing some energy (for example heat, kinetics) to shape this construction. In the bilayer structure, hydrophobic domain is arranged away from the aqueous phase, while the hydrophilic heads stay in touch with the dispersion medium. A niosome comprises of drug, cholesterol or its derivatives, non-ionic surfactants and, in some cases, ionic amphiphiles. The drugs, both hydrophilic and hydrophobic, can be encapsulated in the niosomes. Hydrophilic drugs are encapsulated in the core, while hydrophobic drugs are entangled in the hydrophobic region of the bilayer\(^1\). Niosomal drug delivery is potentially relevant to many pharmacological agents because of their action towards numerous illnesses. To design the novel drug delivery system, it could be used as vehicle for poorly absorbable drugs as well. This enhances the bioavailability through crossing the anatomical barrier of gastrointestinal tract via transcytosis of M cells of Peyer’s patches inside the intestinal lymphatic tissues\(^2\). In recent years, with the improvement of nanotechnologies more and more studies have focused on niosomes as nanocarriers for drug delivery\(^3\).

The niosomes are being used to study the nature of the immune response provoked via antigens due to their immunological selectivity, low toxicity and more stability. Currently, niosomes are getting more attention in topical drug transport because of its wonderful characteristics such as enhancing penetration of medicine, offering a sustained pattern of drug release and capability to carry both hydrophilic and lipophilic drugs\(^4\).

Niosomal drug delivery has been extensively studied for its usage via diverse strategies of administration\(^5\) together with intramuscular\(^6\), intravenous\(^7\), peroral and transdermal\(^8\). Similarly, niosomes as drug delivery vesicles had been shown to enhance absorption of few drugs across cellular membranes\(^9\), to localize in organs\(^10\) and tissues and to elude the reticuloendothelial system. Niosomes have been used to encapsulate colchicine\(^11\), estradiol\(^12\), tretinoin\(^13\), dithranol\(^14\), enoxacin\(^15\) and for application including anticancer, anti-tubercular, anti-leishmanial, antiinflammatory, hormonal drugs and oral vaccines\(^16\). In this paper we have studied impact of process variables like quantity of drug and type of surfactant, cholesterol content, material and price, methods of preparation and resistance to osmotic pressure on niosome formulation.

Spasticity is a condition wherein muscle tissue are continuously contracted inflicting stiffness or tightness which may impede movement and speech. Additionally, it is caused by damage to the spinal cord that controls voluntary movement\(^17\).

Some of the more common situations related to spasticity encompass sclerosis, spinal cord injury, stressful brain injury, cerebral palsy, and put up-stroke syndrome. In many patients with such conditions, spasticity may be disabling and painful, with a marked impact on quality of life\(^18\).
The drugs like baclofen, dantrolene, and tizanidine are considered effective for the treatment of spasticity. These 3 medicines act via specific mechanisms: baclofen blocks pre- and post-synaptic GABAB receptors\(^{19-20}\); tizanidine is a centrally-acting agonist of α2 receptors\(^{21-22}\) and dantrolene inhibits muscle contraction by reducing the discharge of calcium from skeletal muscle sarcoplasmic reticulum\(^{23}\). There are others drugs which have been used to deal with spasticity but no longer officially prescribed for this indication consist of benzodiazepines, clonidine, gabapentin, and botulinum toxin\(^{23-25}\). In this research authors have prepared and evaluated the different span 60 niosomal formulations containing baclofen for skeletal muscle relaxant activity in mice.

### Materials and Methods

Baclofen was provided as gift sample from Sun Pharmaceutical Industries Limited, India. Triton X-100, Span 40, Span 60 and Cholesterol were purchased from Sigma, USA. Diethyl ether and methanol were purchased from E Merck, Mumbai, India. The all other materials used in this study were of analytical grade.

The niosomes were prepared by ether injection method with moderate modification\(^{26,27}\). The surfactant and cholesterol were dissolved in 20 mL of diethyl ether in molar ratio viz. 0.5:1, 1.0:1, 1.5:1, 2.0:1 and 1.0:2 (Table 1). Then the solution so formed was injected slowly via a 14 gauge needle into an aqueous solution (maintained at 60°C) of baclofen (1% w/v). The niosomal suspension was left to mature overnight at 4°C and stored at refrigerator for further studies.

### Table 1. Ratio of surfactant and cholesterol for preparation of niosomes

| Formulation Code | Surfactant | Surfactant: Cholesterol ratio (μ mol) | Surfactant Quantity (mg) | Cholesterol Quantity (mg) |
|------------------|------------|-------------------------------------|--------------------------|---------------------------|
| F1               | Span 40    | 0.5:1                               | 0.194                    | 0.386                     |
| F2               | Span 40    | 1.0:1                               | 0.388                    | 0.386                     |
| F3               | Span 40    | 1.5:1                               | 0.582                    | 0.386                     |
| F4               | Span 40    | 2.0:1                               | 0.776                    | 0.386                     |
| F5               | Span 40    | 1.0:2                               | 0.388                    | 0.772                     |
| F6               | Span 60    | 0.5:1                               | 0.215                    | 0.386                     |
| F7               | Span 60    | 1.0:1                               | 0.431                    | 0.386                     |
| F8               | Span 60    | 1.5:1                               | 0.646                    | 0.386                     |
| F9               | Span 60    | 2.0:1                               | 0.862                    | 0.386                     |
| F10              | Span 60    | 1.0:2                               | 0.431                    | 0.772                     |

### Characterization of niosome

#### Vesicle size

The size of niosomes was determined by using film of vesicle dispersion, fixed with 10% w/v gelatin solution.

It was observed under a light microscope (BEM-21, Besto Microscope, India) fitted with an ocular micrometer and stage micrometer at magnification of 100X. Approximately 100 vesicles were selected at random and their size was measured\(^{28}\).

#### Vesicle shape

Scanning electron microscopy (SEM) (Philips-XL-20, Netherlands) was performed to characterize the surface morphology of niosomal formulations. Niosomes were installed immediately onto the sample stub and covered with gold film (200 nm) under reduced pressure (zero.133 Pa)\(^{28}\).
Entrapment efficiency
The niosomal dispersions were centrifuged (90 XL Ultracentrifuge, Beckman, U.S.A.) at 10,000 × g for 20 min to separate unentrapped drug and washed with phosphate buffered saline (pH 7.4). The clear supernatant was analyzed for baclofen via UV-spectrophotometer (Shimadzu UV-1700, Japan) at 226 nm. The amount of entrapped drug was calculated using the following equation:

In vitro drug release study
The niosomes encapsulating baclofen were separated via gel filtration on sephadex G-50 column in double distilled water for 10 h for swelling. Then the prepared niosomal suspension (1mL) was placed on the top of the column elution and the process was performed using normal saline. The niosomes loaded with baclofen elutes out first as dense, white opalescent suspension. Separated niosomes were filled in dialysis tube to which a dialysis sac was attached to both ends. The dialysis tube was suspended in phosphate buffered saline (pH 7.4) at 37±2°C, stirred with magnetic stirrer and samples were withdrawn at specific time intervals and analyzed spectrophotometrically for baclofen (λmax 226 nm). The volume was replenished with the same amount of fresh dissolution fluid each time to keep the sink situation.

Stability study
The niosomal formulations were subjected to stability studies by storing at 4±2°C, 25±2°C and 37±2°C in thermostatic oven for 6 months. Then drug content of all the formulations was determined in terms of % entrapment at reported temperature.

In vivo study
The apparatus consists of a horizontal wooden rod or steel rod lined with rubber with 3 cm diameter connected to a motor with the speed adjusted to 30 rotations per minute. The rod is 40 cm in length and is split into 3 sections by plastic discs, thereby permitting the simultaneous testing of 125 mice. The rod is at a height of approximately 50 cm above the table top so as to discourage the animals from jumping off the roller. Cages beneath the sections serve to restrict the movements of the animals when they fall from the roller. The experiments were carried out on Swiss albino mice weighing 20-25 g. The mice have been trained to run on the rod rotating at 10 rpm for 300 s. Only those animals which have confirmed their ability to remain at the revolving rod for at least 300 s were used for the test. The animals were divided into 7 groups of six mice each. The drug was administered as shown below:

- Group I – positive control (diazepam 2 mg/kg)
- Group II – plain drug (1-4 mg/kg)
- Group III – niosomal formulation F6 (1-4 mg/kg)
- Group IV – niosomal formulation F7 (1-4 mg/kg)
- Group V – niosomal formulation F8 (1-4 mg/kg)
- Group VI – niosomal formulation F9 (1-4 mg/kg)
- Group VII – niosomal formulation F10 (1-4 mg/kg)

The formulation as well as plain drug were applied topically with the help of custom designed plastic ring. Then it was allowed to be in contact with mouse skin for half an hour to ensure proper absorption. The mice were placed for 5 min at the rotating rod. The number of falls from the roller were counted. Diazepam (2 mg/kg, i.p.) was used as positive control. The data of number of falls was analyzed by one way ANOVA followed by means of Student-Newman-Keuls test. A p value (p<0.001) was considered significant.
Results and Discussion

Vesicle size
Particle size determination of niosomes was carried out with the aid of light microscope after storage. The various ratios of niosomal formulations were taken for size evaluation. The vesicle size of the niosomes was found to be in the range of 3.62±0.54-4.08±0.64 µm (Table 2). The size of the span 60 vesicles was uniform and independent of surfactant.

Table 2. Determination of mean vesicle size of baclofen niosomes composed of span 40 and span 60 (n=3)

| Formulation Code | Mean particle size (µm) |
|------------------|------------------------|
| F1               | 4.05±0.37              |
| F2               | 3.88±0.35              |
| F3               | 3.96±0.42              |
| F4               | 3.81±0.56              |
| F5               | 4.08±0.64              |
| F6               | 3.67±0.25              |
| F7               | 3.72±0.68              |
| F8               | 3.84±0.73              |
| F9               | 3.62±0.54              |
| F10              | 3.92±0.28              |

Vesicle shape
The surface morphology of niosomes was determined by scanning electron microscopy (SEM). The niosomes were smooth, spherical in shape and mostly small multilamellar (Figure 1). The vesicles were isolated and separated with no aggregation or agglomeration.

Figure 1. SEM of optimised niosomal formulation (magnification 100x).

Entrapment efficiency
The entrapment efficiency of formulations F1, F2, F3, F4, and F5 of span 40 and F6, F7, F8, F9 and F10 of span 60 was observed to be 67.43±0.27 %, 76.43±0.31 %, 74.76±0.67 %, 75.82±0.34 % and 72.23±0.15 %, respectively and 78.45±0.82 %, 83.69±0.58 %, 87.72±0.17 %, 88.44±0.28 % and 82.38±0.32 %, respectively. The maximum percent drug entrapment (88.44±0.28%) was observed with formulation F9 of span 60. Increase in cholesterol with surfactant concentration, led to improved percentage drug en-
trapment and beyond this increase in cholesterol concentration had no influence on percentage drug entrainment. From the above study it was observed that as the cholesterol content in the vesicles increased, the incorporation of the drug in the vesicles also increased. Cholesterol improves the fluidity of the bilayer membrane and additionally improves the stability of bilayer membrane in the presence of biological fluids such as blood/plasma (Table 3).

Table 3. Effect of span 40 and 60 on % entrapment efficiency of baclofen niosomes

| Formulation Code | Surfactant type | % EE       |
|------------------|----------------|-----------|
| F1               | Span 40        | 67.43±0.27|
| F2               | Span 40        | 76.32±0.31|
| F3               | Span 40        | 74.76±0.67|
| F4               | Span 40        | 75.82±0.34|
| F5               | Span 40        | 72.23±0.15|
| F6               | Span 60        | 78.45±0.82|
| F7               | Span 60        | 83.68±0.58|
| F8               | Span 60        | 87.72±0.17|
| F9               | Span 60        | 88.44±0.28|
| F10              | Span 60        | 82.38±0.32|

In vitro drug release study

The in vitro drug release studies showed that time taken for drug release was 10 h for niosomes containing baclofen prepared by span 40 and span 60 (Figure 2, 3). The maximum % cumulative drug release from baclofen niosomes in PBS (pH 7.4) at 37±2°C was 78.14±0.66 %, 82.38±0.29 %, 81.38±5.40 %, 81.67±9.36 % and 80.45±5.43 % for formulations F1, F2, F3, F4 and F5 of span 40 respectively (Figure 2), whereas 86.48±4.56 %, 87.78±4.99 %, 84.98±6.80 %, 87.88±8.66 % and 83.45±7.93 % for formulations F6, F7, F8, F9 and F10 of span 60, respectively (table 6). Formulation F9 indicates maximum % drug release (87.88±8.66 %) as compared to F6, F7, F8 and F10 of span 60 formulation (Figure 3). The drug release was apparently low in magnitude (pure drug-about 92 % vs. F9-87 %) and observed for longer period of time as compared to pure drug taken as standard. Therefore, the drug release could be said to be in sustained fashion.

As the cholesterol ratio increased the % cumulative drug release also increased but afterwards it shows diminished release of drug which is evident from previous studies[34-36]. The slow release of drug from multilamellar vesicles may be ascribed to the fact that multilamellar vesicles consist of several concentric sphere of bilayer above the aqueous compartment.

Figure 2. Effect of span 40 concentration on in vitro % release profile of baclofen niosomes in PBS 7.4 pH, 37±2°C at λ max 226 nm (n=3)
Stability study

The stability studies were performed on span 40 and span 60 formulations (Table 4). The % entrapment efficiency values upon storage were 96.65±0.45%, 90.53±0.71% and 85.32±0.49% at 4±2°C, 25±2°C and 37°±2C, respectively, (Table 4) for the F9 formulation and its maximum compared to respective formulations. The % drug entrapment of formulations stored at 4±2°C was highest followed by formulation stored at 25±2°C and 37±2°C. This may be due to phase transition of surfactants and lipid causing leakage from vesicle at higher temperature during storage. Hence, from the data, the optimum storage condition for the baclofen niosomes was 4±2°C. Non-ionic surfactant with cholesterol is suitable carrier for the preparation of niosomes of baclofen. Stability study reveals that span 60 confirmed maximum % drug entrapment efficiency which can be attributed to high lipophilicity of the surfactants.

Table 4. Stability study of different formulations of span 40 and 60 containing baclofen

| Formulation Code | Time (month) |  |  |  |
|------------------|--------------|---------------|---------------|---------------|
|                  | 1            | 3             | 6             |               |
| **Temperature (°C)** |              |               |               |               |
| 4±2              |              |               |               |               |
| **% Entrapment** |              |               |               |               |
| F1               | 78.23±0.21   | 76.61±0.18    | 75.14±0.39    |               |
| F2               | 83.62±0.15   | 82.54±0.33    | 81.50±0.60    |               |
| F3               | 81.14±0.41   | 80.32±0.60    | 78.56±0.76    |               |
| F4               | 80.24±0.30   | 79.26±0.71    | 77.80±0.68    |               |
| F5               | 81.63±0.46   | 81.34±0.38    | 80.67±0.74    |               |
| F6               | 87.76±0.47   | 86.64±0.48    | 85.87±0.56    |               |
| F7               | 92.23±0.13   | 91.38±0.74    | 90.35±0.52    |               |
| F8               | 95.87±0.67   | 93.89±0.75    | 93.56±0.62    |               |
| F9               | 96.65±0.45   | 96.32±0.15    | 95.23±0.83    |               |
In vivo study was performed to test the potential activity of formulation, and it was carried out using rota-rod apparatus at CT Institute of Pharmaceutical Sciences, Jalandhar (Punjab), India. For in vivo study 42 Swiss albino mice were selected. The dosage regimen, was 1 to 4 mg/kg for formulations of span 60 (F6, F7, F8, F9 and F10) and plain drug as per literature. The plain drug treated (1, 2, 3 and 4 mg/kg) mice showed a lesser number of falls from rota-rod apparatus in comparison to span 60 formulations (1, 2, 3 and 4 mg/kg) and diazepam (2 mg/kg), results are given in figure 4-figure 8. Formulation F9 shows the maximum number of falls than the F6, F7, F8 and F10 formulations of span 60. Similarly, optimized formulation F9 treated mice showed an increased number of falls than plain drug treated mice, however a greater number of falls was observed in case of diazepam treated mice. When the dose of optimized formulation was increased (1, 2, 3 and 4 mg/kg) there was an increase in the number of falls from the rota-rod apparatus, because niosomal formulation permeated well via the skin and absorbed into systemic circulation, and may finally relax the muscle. The mice treated with formulations have shown improved muscle relaxant activity which was evident by using increased range of falls in rota-rod test as compared to plain drug treated mice. The data pertaining to the range of falls have been analyzed by using one way ANOVA followed by student-Newman-Keuls test. The effect of formulation was dose dependent (p < 0.001; for 1, 2, 3 and 4 mg/kg, respectively). However, diazepam treated mice may result higher muscle relaxation than any dose of formulation tested (Figure 7).
Figure 4. Comparison between plain drug, formulation F6 and diazepam on albino mice for skeletal muscle relaxant activity.

Figure 5. Comparison between plain drug, formulation F7 and diazepam on albino mice for skeletal muscle relaxant activity.

Figure 6. Comparison between plain drug, formulation F8 and diazepam on albino mice for skeletal muscle relaxant activity.
NOTE: M.D. = Mean deviation; S.D. = Standard deviation; *** = P<0.001

Figure 7. Comparison between plain drug, optimized formulation F9 and diazepam on albino mice for skeletal muscle relaxant activity.

Figure 8. Comparison between plain drug, formulation F10 and diazepam on albino mice for skeletal muscle relaxant activity.

Conclusion
The present investigation has endeavored to formulate and evaluate niosomes containing baclofen. The ether injection method has been used for the preparation of vesicular carriers. The average particle size of formulated niosomes was in the range of 3.62±0.54-4.08±0.64 μm and vesicles were smooth, spherical in shape and mostly small multilamellar. The muscle relaxant activity of the optimized formulation has been found improved as compared to plain drug, thereby suggesting the niosomes as potential drug carrier meant for topical administration.
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