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Preparation and Characterization of Topical Niosomal Formulation Containing Retinyl Palmitate and Squalane for Enhanced Skin Delivery

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ABSTRACT: Retinyl palmitate (RP), also known as vitamin A palmitate, is a well-known anti-aging ingredient that promotes collagen production, enhances skin flexibility, and decreases the view of fine lines and wrinkles. Squalene, a natural lipid found in the skin, is also known to have anti-aging properties, including moisturizing and protecting the skin from environmental stressors. In this study, we demonstrate the encapsulation of RP and squalene within niosomes, and evaluate the *in-vitro* release and skin permeation of these active ingredients. The niosomes were prepared using a thin-film hydration method and characterized for their particle size, polydispersity index, zeta potential, and encapsulation efficiency. The encapsulation efficiencies of optimized niosome formulation for RP and Squalene were determined to be 83.5%±2.55% and 91.2%±5.12%, respectively. Narrow size distribution was accomplished with a particle size of 168.4 ± 8.2 nm. Cumulative release of 82.67%±5.23% for RP and 87.12%±5.65% for Squalene was acquired after 24 hours of *in-vitro* release study in sink conditions. *Ex-vivo* permeation studies indicated that niosomes had a much higher retention rate for RP and Squalene in both the Stratum Corneum (SC) and viable epidermis/dermis layers when compared to the commercial product. Stability studies showed that the optimized niosomes were convenient for keeping at 4°C for at least 90 days. The outcomes show that niosomes are effective in delivering RP and squalene to the skin and can provide a promising alternative to traditional anti-aging formulations.

KEYWORDS: Retinyl palmitate; Squalene; Niosomes; Skin permeation; Epidermal targeting.

1. INTRODUCTION

Skin aging, both intrinsic (chronological aging) and extrinsic (sun exposure, pollution, smoking, etc.), is a complex process that is influenced by multiple factors, including oxidative stress [1]. As the skin is continuously subjected to external stressors, it is more susceptible to oxidative damage than other internal organs. Reactive oxygen species (ROS) can induce inflammation and cause the breakdown of the extracellular matrix, leading to cellular dysfunction, aging, hyperpigmentation and cell death. Additionally, ROS can interfere with the production of collagen and elastin, two essential proteins that give the skin its firmness and elasticity [2,3].Antioxidant molecules are highly effective in addressing the issues brought on by oxidative stress in the skin, including increasing collagen production. To combat oxidative stress and skin aging, it is crucial to limit exposure to environmental stressors, maintain a healthy diet rich in antioxidants, and use topical products that contain antioxidants and other anti-aging ingredients. Additionally, practicing good skin care habits, such as using sun protection and moisturizer, can help to decelerate the aging process [4].

Squalene is a natural compound found in high concentrations in the skin, particularly in the sebum produced by the sebaceous glands. It is thought to contribute to maintaining the integrity and elasticity of the skin [5,6]. It is also a potent antioxidant and has been shown to have anti-aging properties when applied topically to the skin. However, there have been some reports of adverse effects associated with using squalene on the skin. One potential adverse effect is contact dermatitis, which is a type of skin irritation caused by an

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allergic reaction to a particular substance. Another potential adverse effect is acne. Research has demonstrated that Squalene can enhance sebum production, potentially resulting in acne formation [7,8].

Retinyl palmitate (RP), also known as vitamin A palmitate, is a form of vitamin A that is commonly used in skincare products. RP has gained recognition for its ability to combat aging, as it can decrease the visibility of lines and wrinkles, improve skin texture, and even out skin tone. It also has antioxidant characteristics that can prevent the skin from harm caused by external conditions like ultraviolet radiation and contamination [9,10]. Additionally, RP promotes the creation of extracellular matrix proteins like collagen by fibroblasts found in the dermis. Changes in these levels can lead to a breakdown of the layers of corneocytes and subsequently, an increase in transepidermal water loss [11,12]. However, it can also be irritating to the skin, causing redness, dryness, and flaking in some individuals. It is important to use a low concentration of RP and gradually increase the frequency of use to minimize these side effects [13]. Consequently, designing a suitable drug delivery system that ensures the efficient delivery of RP and squalene within the skin while minimizing potential side effects is crucial.

Niosomes are spherical vesicles made of non-ionic surfactants that can be used to deliver a variety of substances, including hydrophilic and lipophilic active ingredients, to the skin [14,15]. Niosomes have been shown to have several potential benefits when used in skincare products, including enhanced stability and permeation of the active compounds, which can be beneficial for the therapy of certain skin problems such as acne and eczema and reduced irritation compared to conventional dosage forms. Additionally, niosomes have been found to be higher degree of stability and have a longer shelf life than some other drug delivery systems, making them a potentially viable option for long-term skin treatment [16,17]. Overall, niosomes appear to be a promising alternative for the delivery of a variety of compounds to the skin, and further research is needed to fully understand their potential and limitations.

The purpose of this research was to produce squalene and RP loaded niosome for preparing antiaging formulations. Niosomes were made using a technique known as thin-film hydration and were then homogenized using sonication. Physicochemical characterization and stability tests were performed on niosomal formulation. *Ex-vivo* permeation tests were implemented to evaluate the permeation capacity of the formulations.

2. RESULTS AND DISCUSSION

2.1. Particle size, polydispersity index and zeta potential

Particle size, polydispersity index and zeta potential values of niosomal vesicles were measured. As shown in Table 1, the size of the niosomes were varied between 152.6 ± 5.5 nm and 423.2 ± 12.4 nm. The particle size results showed that when the quantity of surfactant was raised from 25 to 50 mg, there was a noticeable increase in vesicle size. However, when the amount was further increased from 50 to 100 mg, the size of the niosomes decreased. The findings of our research matched those from previously conducted studies [18,19]. An increase in cholesterol levels has been observed to correspond with an increase in the size of niosomal vesicles. This is because when cholesterol is added to the formulation, it can embed itself in the lipid bilayer with its aliphatic chain aligned parallel to the hydrocarbon chains in the middle of the bilayer and its hydrophilic part facing the aqueous layer. This results in the lipid bilayer becoming more hydrophobic, which causes disruption in the integrity of the vesicular membrane and leads the vesicles to become larger [20].

The zeta potential measuring of the surface charge of colloidal systems, reveals information about the characteristics of niosomes present in the dispersion medium. Additionally, it is frequently utilized to determine the stability of vesicular system. In this study, the zeta potential values of prepared niosomes were between -15.3 ± 0.4 to -19.2 ± 0.2 mV, as can be seen in Table 1. The findings support a previous study by Basiri et al, who determined that niosomal formulations made with Tween 60/Span 60 have a negative charge surface [21].

The measurement of the polydispersity index using dynamic light scattering provides information on the distribution of particle sizes. It is crucial that this value is kept low to prevent aggregation from occurring in the niosomal system over time and hence maintain stability. Table 1 shows that the polydispersity index for all niosomal systems ranges from 0.12 to 0.42, indicating that niosomal systems provide an appropriate size distribution and a narrow dispersity.

Table 1. Characterization of Niosomes containing RP and Squalene

Formulation	Particle Size	Polydispersity Index	Zeta Potential	Encapsulation Efficiency(%)RP	Encapsulation Efficiency(%)Squalene
F1	152.6 ± 5.5	0.24±0.08	-15.3 ± 0.4	56.2	61.3
F2	295.1 ± 10.1	0.42±0.07	-19.2 ± 0.2	79.1	84.6
F3	168.4 ± 8.2	0.12±0.02	-16.7 ± 0.8	83.5	91.2
F4	250.3 ± 7.2	0.16±0.05	-16.2 ± 0.5	45.8	52.6
F5	423.2 ± 12.4	0.38±0.22	-15.9 ± 0.3	61.7	74.3
F6	304.6 ± 16.8	0.29±0.16	-18.3 ± 0.7	77.5	85.9

2.2. Determination of Encapsulation Efficiency

As shown in Table 1, the encapsulation efficiency of RP in niosome formulations ranged from 45.8% and 83.5%. And also, the encapsulation efficiency of squalene was detected to be between 52.6% and 91.2%. The encapsulation efficiency value in niosome formulations indicated a tendency to increased when the concentrations of surfactants were increased. This is because surfactants contain the longest saturated alkyl chain and as their amount increases, more active ingredients are able to be encapsulated in the available area of the surfactant. The enhance in the available hydrophobic area of the niosomal system allows for the entrapment of more hydrophobic drugs [18,22]. However, when the amount of cholesterol increases, the encapsulation efficiency values decrease. This is most likely caused by the restricted space of the hydrophobic area in the bilayer membrane and the possibility that cholesterol, when used in high amounts, replaces the hydrophobic drug in the bilayer. Our findings matched those from previous studies [23,24]. Since F3 formulation has maximum encapsulation efficiency value for the both active ingredients and the optimum particle size, it was determined as the optimum formulation and further studies were continued on this formulation.

2.3. *In-vitro* release study

Using the dialysis membrane diffusion method, the drug-release characteristics of the produced niosomal formulations were evaluated. Release behaviors of RP and Squalene from niosomal formulation were given in Figure 1. The results indicated that there was a initial burst of release for RP and Squalene, with 50.2% and 56.8% being released within the first 2 hours, respectively. This was followed by a slower release phase. It was determined that the lipophilic drugs were located within the bilayer membrane or on the surface and outer layers of the niosomes. The first release is believed to be caused by the detachment of the active ingredients from the surface, while the release is a result of sustained release of the hydrophobic active ingredients encapsulated within the bilayer membrane and inner layers [25]. After 24 hours, a cumulative release of 82.67%±5.23% for RP and 87.12%±5.65% for Squalene was observed. The release data for both RP and Squalene was analyzed using different models to determine the release mechanism. The use of mathematical modeling to evaluate the release kinetics of drugs from dosage forms is crucial in understanding the physical processes that affect active ingredients release. The correlation coefficients(r^2) obtained from the release study were used to determine the release mechanism and it was found that the RP and Squalene were released from the niosomal formulations following the Higuchi kinetics model (Table 2). The release of RP and Squalene from the niosomal system is based on the diffusion process described by Fick's law, which means that it depends on the square root of time. These findings align with former research [26,27].

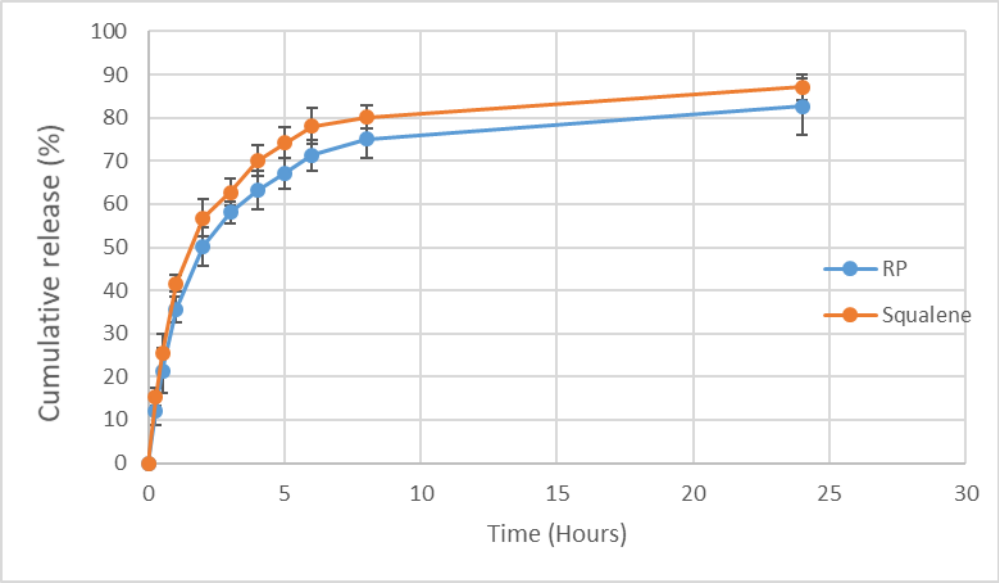

Figure 1. *In-vitro* release profile of F3 formulation

Table 2. Regression values of kinetic release models of the optimized formulations

Kinetic Models	RP	Squalene
Zero-order (r^2)	0.8919	0.8617
First-order (r^2)	0.9255	0.9535
Hixson–Crowell (r^2)	0.9369	0.9287
Higuchi (r^2)	0.9862	0.9834

2.4. *Ex-vivo Permeation Studies*

The process of dermal permeation, which is necessary to achieve local effect, involves the release of drug from the niosomal system, permeation through the SC and targeting the viable epidermis/dermis layers. The effectiveness of permeation depends mainly on the physicochemical characteristic of the active compounds and the vehicles. The purpose of this research was to increase the concentration of active ingredients in the outer layers of the skin and prevent them from entering the bloodstream. During the permeation experiments, neither RP nor Squalene were present in the receptor medium at the conclusion of the 6-hour experiment. The amounts of RP and Squalene accumulated in the skin after 6 hours of application were shown in Figures 2 and 3, for the SC, viable epidermis/dermis layers, respectively. According to the results of *ex-vivo* skin permeation studies, it was determined that the active substances in the prepared niosomes permeated significantly more in both the SC and viable epidermis/dermis layers compared to the commercial product ($p<0.01$).

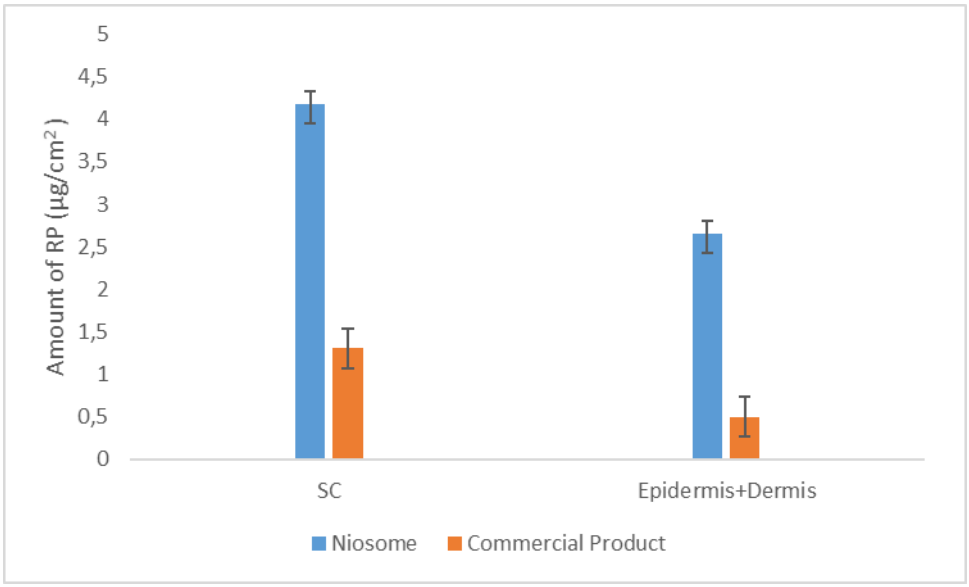

Figure 2. RP amount permeated in SC and viable epidermis/dermis layers.

The findings revealed that niosomes had a retention rate that was 3.21 and 5.41 times greater than that of the commercial product for RP in the SC and viable epidermis/dermis layers, respectively. Additionally, the results demonstrated that niosomes had a retention rate that was 3.55 and 5.24 times greater than that of the commercial product for Squalene in the SC and viable epidermis/dermis layers, respectively. It is possible that the reason for these results is the small particle size of the optimized niosomal formulation, which increases the surface area and allows for better permeation and retention of the active ingredients within the skin [18,28].

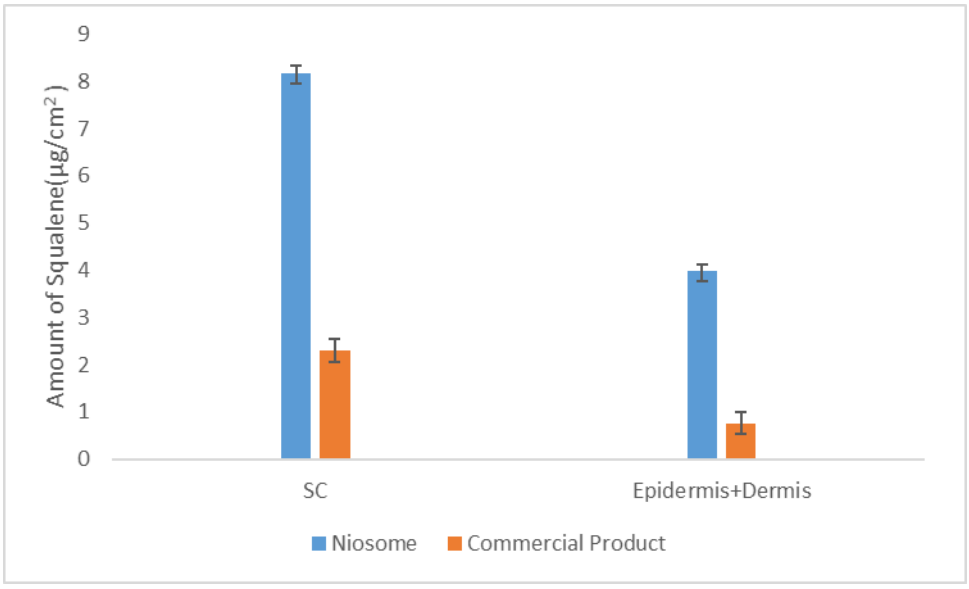

Figure 3. Squalene amount permeated in SC and viable epidermis/dermis layers.

2.5. *Stability Studies*

To ensure the stability of the optimized niosomes, an analysis was conducted by storing them in a refrigerator at 5±3 °C and 25±2 °C at 60%±5% relative humidity for 90 days. Different physicochemical properties were observed to assess the stability of the niosomes, such as particle size, zeta potential, polydispersity index and physical controls. It was found that the niosomes stored at 25±2 °C and 60%±5% relative humidity conditions indicated sedimentation caused by an increase in particle size, thus the stability analysis was not maintained at this temperature. However, when the niosomes were stored in a refrigerator

at 5±3 °C, the particle size, zeta potential and polydispersity index were assessed and it was found that no statistically significant variations were observed in Table 3 ($p > 0.05$). The outcomes showed that optimized niosomes were found to maintain its stability for 90 days at 5±3 °C.

Table 3. The result of stability studies of optimum niosomal formulation

Time (days)	Particle Size (nm)	Zeta Potential(mV)	Polydispersity Index
0	168.4 ± 8.2	-16.7 ± 0.8	0.12±0.02
30	172.1 ± 12.7	-18.5 ± 2.1	0.14±0.03
60	155.8 ± 9.3	-15.1 ± 1.5	0.13±0.06
90	179.6 ± 10.1	-16.2 ± 3.5	0.17±0.08

3. CONCLUSION

In this research, niosomal systems incorporating both RP and Squalene were successfully formulated for the first time and optimized to reach high encapsulation efficiency values and low particle size. The niosomes were prepared through the process of thin-film hydration and then subjected to sonication. Particle size, polydispersity index and zeta potential values of the prepared niosomes were suitable for topical administration of drugs. It was discovered that niosomes remained stable for a period of 90 days when stored at a temperature of 4°C. The results of the *in-vitro* release and *ex-vivo* permeation studies indicated an extended release of the active ingredients and improved permeation. Therefore, it can be suggested that the combination of RP and Squalene in a niosome structure is a beneficial method for achieving extended efficacy and simultaneous administration of both active compounds. Further studies are necessary to completely comprehend the capabilities of this combination, but the results so far suggest that niosome containing RP and squalene may be a valuable addition to any anti-aging skin care routine.

4. MATERIALS AND METHODS

4.1. Materials

RP, Squalene, Tween 60, Span 60 and cholesterol (CH) were ensured from Sigma– Aldrich, Inc. (St Louis, MO, USA). Dialysis membrane (12-14 kDa, Spectra/Por) was purchased from Repligen. The all chemicals and reagents used in this study were of analytical quality. A commercial product in the form of serum containing retinyl palmitate and squalene, The NewLab.® was used as a control in *ex-vivo* skin permeation study.

4.2. Methods

4.2.1. Preparation of Niosomes

A type of vesicular system called niosomes, which is made from a non-ionic surfactant, was created using a thin film hydration technique [29,30]. Tween 60 and Span 60 were used as non-ionic surfactants. The process involved dissolving a RP, Squalene, Tween 60, Span 60 and cholesterol in chloroform and then evaporating the chloroform under reduced pressure at 40°C using a rotary evaporator. The thin film that was produced was then hydrated at 60°C with an aqueous phase and subjected to sonication. The prepared dispersion after hydration was sonicated using a probe sonicator at 45% amplitude for 10 min (prob; MS 73, Bandelin Sonopuls HD 2070, Berlin, Germany) to decrease the particle size of niosome formulations containing RP and Squalene. The ingredients of niosome formulations are displayed in Table 4. The optimum niosome formulation was determined by considering the particle size and encapsulation efficiency values of the obtained niosomes.

Table 4. The Composition of Niosomes containing RP and Squalene

Code	RP(%)	Squalene(%)	Span 60(1:1)Tween 60 (mg)	Cholesterol (mg)	PBS pH 7.4(mL)
F1	0,3	10	25	20	10
F2	0,3	10	50	20	10
F3	0,3	10	100	20	10
F4	0,3	10	25	40	10
F5	0,3	10	50	40	10
F6	0,3	10	100	40	10

4.2.2. Encapsulation efficiency of niosome vesicles

The encapsulation efficiencies of niosomes were determined using the centrifugation method. The all niosome formulations was centrifuged at 20000 g for 1 hour to distinct the encapsulated drug from the unencapsulated drug [31]. The non-encapsulated drug and free surfactants in the supernatant were removed, diluted with acetonitrile, and quantified using the validated reversed-phase high-pressure liquid chromatographyn (RP-HPLC) technique. For further examination, the niosomal pellet was redispersed with PBS. The following equation was used to calculate the encapsulation efficiency values:

$$Encapsulation\ Efficiency\% = \frac{\text{the amount of total drug} - \text{the amount of non-encapsulated drug}}{\text{the amount of total drug}} * 100$$

4.2.3. Particle size, polydispersity index and zeta potential analyses

Particle sizes and polydispersity index of niosomes were determined by a dynamic light scattering method using Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, UK). With the use of customized cuvettes and the laser Doppler micro-electrophoresis technique, the charge on the surface of the niosomal vesicles, also described as the zeta potential, was determined (Malvern Instruments Ltd). To avoid any effects of multiple scattering, the niosomal formulations were diluted (1:50) with deionized water. Each analysis were conducted at room temperature and repeated a minimum of three times.

4.2.4. Analytical method for RP and Squalene determination

RP-HPLC (Shimadzu LC20-AT; Shimadzu, Kyoto, Japan) instrument combined with a UV-Vis detector was used to measure the quantities of RP and squalene utilizing two distinct techniques. Mobile phases were made out of filtered and degassed mixtures of acetonitrile:ultrapure water (85:15, v/v) for RP and methanol:ultrapure water (70:30, v/v) for squalene. The mobile phase was introduced at a constant flow rate of 1 ml/min by GL Sciences InertSustain C_{18} column that was kept at a temperature of 25 ^{o}C and had dimensions of 250 × 4,6 mm and a particle size of 5 µm. The UV detector was tuned to detect RP at 325 nm and squalene at 196 nm.

The validation of the HPLC method for both RP and Squalene was carried out according to the guidelines set by the International Conference of Harmonization (ICH) Q2 (R1). The stock solution of RP and squalene was used to construct standard curves in the 0.01–5 mg/mL range, which were then analyzed for linearity testing. The results showed a strong linear correlation between the peak area and the concentrations of RP (r^2 = 0.9995) and Squalene (r^2 = 0.9992) within the specified range. Additionally, the samples of free niosomes did not affect the RP and Squalene peaks, indicating the specificity of the method. The RP-HPLC method was also confirmed to be accurate, precise, and stable through further validation.

4.2.5. *In-vitro* release study

The dialysis technique was used to study the release of RP and Squalene from niosome formulations [32]. Before the experiment, cellulose-based dialysis membranes with a molecular weight cutoff of 12–14 000 Da were thoroughly cleaned and submerged in distilled water to remove the preservative. Niosomes (1 ml) were put inside dialysis bags that were then closed with conventional closures. The dialysis bags were submerged in 150 mL of a PBS:Ethanol solution (1:1; pH 7.4). The test was conducted in a shaking incubator at 32°C±0,5°C and 400 rpm. 1 mL of samples were taken from the receptor phase at predefined intervals (0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8 ve 24 hours) and supplied with an equivalent volume of fresh medium to maintain sink conditions. The amount of drug released was analyzed by a validated RP-HPLC method.

Different mathematical models, including zero-order [33], first-order [34], Higuchi root-square [35] and Hixson–Crowell [36] were applied to investigated the kinetic mechanisms of drug release from niosomes as expressed in the equations below.

Zero-order: $C=k_0t + C_0$

First-order: $In\ C= In\ C_0 +k_1t$

Higuchi model: $C= k_2t^{½}$

Hixson–Crowell: $W_0^{1/3} –W_t^{1/3} = k_H$

In these equations C represents the concentration of the active ingredients that has been released at a specific time, t. C_0 represents the initial concentration of the active ingredients in the formulation. The constants k_0, k_1 and k_2 represent the rate of release for zero-order, first-order, and Higuchi release kinetics, respectively. W_0 represents the initial amount of active ingredients in the formulation, while W_t represents the amount of active ingredients remaining in the formulation at a specific time, t; k_H is a constant incorporating the surface-volume relation.

Finally, by analyzing the correlation coefficients, the ideal active ingredients release kinetics from the niosomes were identified.

4.2.6. *Ex-vivo skin permeation study*

The *ex-vivo* skin permeation study was analyzed by utilizing the tape-stripping method to investigate the influence of topical formulations on active ingredients permeation in the skin [37]. Ear skins of 6-month-old pigs used in these studies were obtained from the local slaughterhouse, and the dorsal skin was removed from the cartilage using a scalpel, and homogeneous sections were taken. Before use, the hairs on the skin were cleaned with the help of surgical scissors without damaging the SC layer, and the fat and subcutaneous tissues on the skin were carefully separated with a scalpel. Pig skins were preserved at -20°C until used. The skins removed from the freezer were kept at room temperature for a while before use. Before starting the experiment, the skins were kept in PBS solution for 30 minutes to ensure hydration. In this study, a double-jacketed modified Franz diffusion cell with a receptor phase volume of 7 ml and a surface area of 1.77 cm2 where diffusion occurred was used. (The Hanson Vertical Diffusion Cell). As the receptor phase; Ethanol 1:1 PBS(pH:7,4) solution, which is the medium in which active ingredients dissolve and sink condition is provided, was used. Subsequently, pig skins were carefully placed at the interface between the donor and receptor compartments so that the epidermal side was facing the donor compartment. Optimum niosome formulation and commercial products containing equal amounts of active ingredients with niosome for comparison were applied on the skin. The receptor phase was mixed at 400 rpm using a magnetic stirrer. The diffusion cells were maintained at a constant temperature of 37 ± 0.5 °C. 0.5 ml samples were taken from the receptor phase at the specified times and filtered through a 0.45 µm syringe filter. In order not to decrease the volume of the receptor phase, the same temperature and the same volume of solvent mixture were added to the apparatus. After 6 hours, the experiment was terminated.

After the designated sampling period, the skin samples were removed from the apparatus, rinsed with a solution of PBS, and dried with cotton wool. Adhesive tape (Corneofix) strips were pre-prepared and used to remove the SC by applying gentle pressure and rolling the tape onto the skin surface, repeating the process 20 times. The tape strips and remaining skin samples were then put into acetonitrile for 24 hours and shaken, followed by sonication and centrifugation for extraction. The validated HPLC method was used to examine and measure extract aliquots.

4.2.7. *Stability studies of niosomes*

The formulations were kept at 5±3°C and 25±2°C under 60%±5% relative humidity conditions for 90 days in order to determine the stability of the optimum niosomes. At specific intervals during this time, samples were taken and tested for particle size, polydispersity index, zeta potential and physical characteristics. These measurements were conducted multiple times to ensure accuracy.

4.2.8. *Statistical Analysis*

Each experiment was performed at least three times and the results were then given as the mean±standard deviation (SD). One-way ANOVA was used for the statistical analysis along with the Tukey's *post hoc* test. A difference with p<0,05 was considered to be statistically significant.

Author contributions: Concept – A.A.S.; Design – A.A.S.; Supervision – A.A.S.; Resources – A.A.S.; Materials – A.A.S.; Data Collection and/or Processing – A.A.S.; Analysis and/or Interpretation – A.A.S.; Literature Search – A.A.S.; Writing – A.A.S.; Critical Reviews – A.A.S.

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