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Original Article

Advance in transdermal delivery of calcitonin using nanostructured lipid carrier-based emulgel

[Avance en la administración transdérmica de calcitonina mediante un emulgel nanoestructurado basado en un portador lipídico]

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Abstract

Context: Peptide-protein drugs have a very important role as therapeutic agents for various diseases. However, their therapeutic use has many barriers to delivery, such as large molecular weight, reduced stability during the manufacturing process and storage, and poor absorption when administered orally. One of peptide-protein drugs is calcitonin, a polypeptide of 32 amino acids used to overcome high levels of calcium in the blood, such as hyperparathyroidism. Nevertheless, drug delivery is still challenging to develop.

Aims: To evaluate a calcitonin nanostructured lipid carrier-based emulgel, which could penetrate through the stratum corneum, and meet the stability requirements.

Methods: Four formulas of calcitonin nanostructured lipid carrier (NLC) were prepared by the double emulsion-evaporation method, then all formulas were characterized in terms of particle size, polydispersity index, zeta potential, entrapment efficiency, and particle morphology. Calcitonin NLCs were then formulated into NLC-based emulgel. Further, *in vitro* penetration and stability of NLC calcitonin emulgel studies were conducted.

Results: The 75:1 ratio of total lipid to the drug (F2) was optimal for calcitonin-loaded NLC with a particle size of 135.6 nm, an index polydispersity of 0.1, the zeta potential of 34.7 mV, and an entrapment efficiency of 99.6%. According to the percutaneous penetration study, the calcitonin NLC-based-emulgel resulted in a fivefold enhancement compared to the non-NLC calcitonin emulgel. Moreover, the stability study illustrated calcitonin levels after six months were 46.09-60.95% and 43.45-68.59% at storage conditions of 5 ± 3°C and 25 ± 2°C/RH 60 ±5 %, respectively.

Conclusions: The calcitonin NLC-based-emulgel produced a fivefold enhancement permeation through the stratum corneum.

Keywords: calcitonin; emulgel; nanostructured lipid carrier; peptide-protein drug; transdermal.

Resumen

Contexto: Los fármacos péptido-proteicos desempeñan un papel muy importante como agentes terapéuticos para diversas enfermedades. Sin embargo, su uso terapéutico presenta muchas barreras para su administración, como un gran peso molecular, una estabilidad reducida durante el proceso de fabricación y el almacenamiento, y una absorción deficiente cuando se administran por vía oral. Uno de los fármacos péptido-proteicos es la calcitonina, un polipéptido de 32 aminoácidos que se utiliza para superar los niveles elevados de calcio en sangre, como en el hiperparatiroidismo. Sin embargo, la administración del fármaco sigue siendo difícil de desarrollar.

Objetivos: Evaluar un emulgel nanoestructurado a base de un portador lipídico de calcitonina, que pueda penetrar a través del estrato córneo y cumplir los requisitos de estabilidad.

Métodos: Se prepararon cuatro fórmulas de portador lipídico nanoestructurado (NLC) de calcitonina por el método de doble emulsión-evaporación, y luego se caracterizaron todas las fórmulas en términos de tamaño de partícula, índice de polidispersidad, potencial zeta, eficiencia de atrapamiento y morfología de partícula. A continuación, las NLC de calcitonina se formularon en emulgeles basados en NLC. Además, se realizaron estudios de penetración y estabilidad *in vitro* del emulgel de NLC de calcitonina.

Resultados: La relación 75:1 entre el lípido total y el fármaco (F2) fue óptima para la NLC cargada de calcitonina, con un tamaño de partícula de 135,6 nm, un índice de polidispersidad de 0,1, un potencial zeta de 34,7 mV y una eficiencia de atrapamiento del 99,6%. Según el estudio de penetración percutánea, el emulgel de calcitonina basado en NLC quintuplicó la eficacia del emulgel de calcitonina sin NLC. Además, el estudio de estabilidad ilustró que los niveles de calcitonina después de seis meses eran del 46,09-60,95% y del 43,45-68,59% en condiciones de almacenamiento de 5 ± 3°C y 25 ± 2°C/RH 60 ± 5%, respectivamente.

Conclusiones: El emulgel a base de NLC de calcitonina produjo una permeación cinco veces mayor a través del estrato córneo.

Palabras Clave: calcitonina; emulgel; fármaco péptido-proteico; portador lipídico nanoestructurado; transdérmica.

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INTRODUCTION

Calcitonin is a polypeptide hormone composed of 32 amino acids and produced by parafollicular cells in the thyroid gland (C cells) (Felsenfeld and Levine, 2015; Patton and Borshoff, 2018). Besides being naturally produced by humans, calcitonin can also be made in synthetic forms, such as synthetic analogs of salmon, pork, and eel, which are widely used in the treatment of diseases related to elevated blood calcium levels (hypercalcemia) due to hormonal dysfunction. One of the hormonal dysfunctions that can cause hypercalcemia is hyperparathyroidism (Wimalawansa, 2018). The role of calcitonin in the treatment of hyperparathyroidism is as an agent that inhibits bone remodeling by osteoclasts, which are triggered by high levels of parathyroid hormone (Findlay and Sexton, 2004). Patients with hyperparathyroidism usually have normal blood calcitonin levels. Although hypercalcemia may trigger increased calcitonin levels, these values return to normal before the hypercalcemia is fully resolved (Felsenfeld and Levine, 2015). Since the body does not produce enough calcitonin, external calcitonin is needed to treat the problems associated with hyperparathyroidism.

Currently, calcitonin (salmon) has been widely studied on various delivery routes, such as peroral, parenteral, intranasal, and per-rectal (Torres-Lugo and Peppas, 2000). In oral administration, calcitonin is prone to degradation caused by protease enzymes in digestion and becomes unstable or damaged at environmental pH levels below 3, with a known bioavailability of less than 0.1%. Another alternative delivery method of salmon calcitonin that still needs further development is transdermal. However, since salmon calcitonin is an active substance with a large molecular weight (3431.9 Da), this causes the penetration of salmon calcitonin to be very low (16% at the sixth hour) and is a challenge that still needs to be explored further (Manosroi et al., 2013).

Many approaches are used to overcome stratum corneum permeability barriers in percutaneous delivery, such as formulating active substances on lipid-based nanoparticle carriers, commonly called lipid nanocarriers. One of them is a nanostructured lipid carrier (NLC) (Mendes et al., 2019). In transdermal delivery, NLC has the advantage of preventing crystal formation, improving particle movement, and drug penetration through the stratum corneum (Svarc and Hermida, 2020; Vitorino et al., 2015; Zeb et al., 2019). Penetration of NLC particles can be achieved through passive diffusion mechanisms by altering the structure, thermodynamic properties, and barrier components of the stratum corneum (Gu et al., 2018).

Currently, studies related to the transdermal delivery of salmon calcitonin in the NLC systems have not been conducted. Hence, this study will create transdermal salmon calcitonin in the NLC system, which can assist penetration through the stratum corneum. The salmon calcitonin NLCs were then characterized by particle size analysis, zeta potential, entrapment efficiency, and vesicle morphology. Then, the salmon calcitonin NLCs were formulated into an emulgel dosage form for topical preparation. Since salmon calcitonin is sensitive to temperature, pH, and structural damage, this polypeptide is one of the most challenging to formulate (Chang et al., 2003). The instability of peptide proteins during the formulation process can result in changes in product properties and may cause a reduction or loss of potency and pharmacological effects (Surini et al., 2020). In this study, the calcitonin-loaded NLCs were fabricated and characterized, then followed by the formulation of the NLC based-emulgel. The percutaneous permeation enhancement of the calcitonin NLCs-based emulgel was evaluated via *in vitro* penetration study through stratum corneum. In addition, the stability study of the calcitonin NLCs-based emulgel was conducted.

MATERIAL AND METHODS

Chemical and reagents

Salmon calcitonin was purchased from Salus Nutra Inc. (Guangzhou, China). Softisan 378 and Miglyol 810 were obtained from IOI Oleo GmbH (Hamburg, Germany). Sodium cholate and salmon calcitonin standards were purchased from (Sigma). Poloxamer 188 (BASF Lousiana, United States) was a gift from PT. Megasetia Agung Kimia. Sepigel 305 (Seppic, Paris, France), methyl/propylparaben and NaEDTA (Merck), propylene glycol (Qingdao Aspirit Chemical, Qingdao, China), acetonitrile and methanol HPLC grade (Merck), trifluoroacetic acid (Merck), dichloromethane (Merck), and all other solvent and reagents used were analytical grade.

Salmon calcitonin-loaded NLCs

The salmon calcitonin-loaded NLCs were prepared by the double emulsion-evaporation method. The formulations are presented in Table 1. At first, salmon calcitonin was dissolved in a 5% acetic acid solution (pH 3.3) and stored in the refrigerator. Then, make the aqueous phase by dissolving sodium cholate in distilled water. A salmon calcitonin solution is added to the sodium cholate solution and homogenized using a vortex mixer. In the oil phase,

Table 1. Formulation of salmon calcitonin-loaded NLC.

Material	Concentration (% w/v)			
	F1 (150:1)	F2 (75:1)	F3 (50:1)	F4 (37.5:1)
Calcitonin (salmon)	0.02	0.04	0.06	0.08
Softisan 378	2.25	2.25	2.25	2.25
Miglyol 810	0.75	0.75	0.75	0.75
Sodium cholate	4.5	4.5	4.5	4.5
Dichloromethane	10	10	10	10
Poloxamer 188	0.5	0.5	0.5	0.5
Acetate buffer pH 5	Ad 100	Ad 100	Ad 100	Ad 100

Softisan 378 and Miglyol 812 were dissolved in dichloromethane, then dispersed both phases using an Edmund Buhler homogenizer at 15,000 rpm for 15 minutes to form a water-in-oil (W/O) primary emulsion. After both phases were dispersed, the mixture was sonicated with a sonicator in a water bath for 3 minutes with an amplitude of 30%. Once sonicated, the emulsion was added to a 0.5% poloxamer solution and homogenized at 15,000 rpm homogenizer for 20 minutes until a double emulsion (W/O/W) was formed. After the homogenization step was completed, another sonication was performed for 3 minutes at 30% amplitude. The formed nanoparticles were continued with solvent evaporation using a magnetic stirrer at 35°C for 2 hours. The formed NLCs were then hydrated by adding an acetate buffer solution, sonicated for 3 minutes at 30% amplitude, and stored in a refrigerator (Chen et al., 2013; Garcia-Fuentes et al., 2005). Each formula experiment was conducted in triplicate.

Characterization of salmon calcitonin-loaded NLCs

Particle size and zeta potential

Particle size distribution and polydispersity index of the salmon calcitonin-loaded NLCs were measured using a Zetasizer Z290 (Malvern UK). The prepared NLCs were then diluted (1:200) in distilled water and put into a cuvette. Measurements were taken at 25°C room temperature, with three replicates.

Morphology of the NLCs

The morphology of the salmon calcitonin-loaded NLCs particles was observed using Transmission Electron Microscopy (TEM, Tecnai 200 kV D2360 SuperTwin Microscope, Thermo Fisher Scientific, USA). The prepared NLC dispersions were placed on a carbon-coated grating and analyzed at a lens magnification of 29,000-145,000 times (Leonyza and Surini, 2019).

Entrapment efficiency

The entrapment efficiency of the salmon calcitonin-loaded NLCs was tested using the indirect method. At first, the prepared NLC was added with 0.1 M HCl solution, then centrifuged at 13,000 rpm for 15 minutes. The temperature was maintained at 6°C to minimize the degradation of salmon calcitonin. After centrifugation, the NLC would be separated into sediment and supernatant. The supernatant was taken for 20 µL. Then methanol was added up to 1 mL, dispersed using a vortex mixer, and filtered using a 0.45 µm filter membrane. The concentration of salmon calcitonin in the supernatant was analyzed using HPLC at a wavelength of 215 nm (Chen et al., 2013).

Preparation of the salmon calcitonin NLC-based emulgels

The composition of the salmon calcitonin NLC-based emulgels is presented in Table 2. The emulgel was prepared using Sepigel 305 and Na-EDTA solution, and then the mixture was stirred until homogeneous. Finally, the salmon calcitonin-loaded NLCs were added to the emulgel (Surini et al., 2020).

In vitro penetration test

The *in vitro* penetration study of the salmon calcitonin NLC-based emulgels was evaluated using a Franz diffusion cell. The permeation membranes were retrieved from the abdominal skin of Sprague Dawley male rats weighing 200-250 grams. The retrieved skin was soaked for 12 hours in NaCl 0.9% physiological solution and stored at 4°C (Martihandini et al., 2021). The handling method of the experimental animals was approved by the Health Research Ethics Committee of the Faculty of Medicine University of Indonesia on October 04, 2021, with the ethical

Table 2. Formulation of the salmon calcitonin NLCs-based emulgels.

Material	Concentration (% w/v)				
	EF1	**EF2**	**EF3**	**EF4**	**Non-NLC emulgel**
NLC	NLC F1 equals 100 µg/g sCT	NLC F2 equals 100 µg/g sCT	NLC F3 equals 100 µg/g sCT	NLC F4 equals 100 µg/g sCT	-
Salmon calcitonin solution	-	-	-	-	Equal 100 µg/g sCT
Sepigel 305	6	6	6	6	6
Propylenglycol	7	7	7	7	7
Na_2EDTA	0.05	0.05	0.05	0.05	0.05
Methyl paraben	0.1	0.1	0.1	0.1	0.1
Propyl paraben	0.05	0.05	0.05	0.05	0.05
Aquadest	Ad 100	Ad 100	Ad 100	Ad 100	Ad 100

approval number KET-964/UN2.F1/ETIK/PPM.00.02/2021.

The skin membrane was placed into the diffusion chamber between the donor and receptor compartments, with the stratum corneum facing the donor area. The effective area for this penetration test was 1.77 cm^2, and the volume of the receptor compartment was ±7 ml with the temperature maintained at 37°C. The salmon calcitonin NLC-based emulgel was placed on the skin surface in the donor compartment. The phosphate buffer or medium in the receptor compartment was continuously stirred using a magnetic stirrer at 300 rpm with the temperature maintained at 37°C. One ml of sample was taken through the sampling section of the receptor compartment at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 hours. Each time a sample is taken, the phosphate buffer medium must be replaced with the same volume. The collected samples were then analyzed quantitatively using HPLC at a wavelength of 215 nm. Calculation of flux and permeability coefficient can be calculated using the following equations [1] and [2].

$$J = \frac{dQ}{dt} \cdot A \quad [1]$$

$$Cp = \frac{\text{Steady-state flux}}{\text{Donor concentration}} \quad [2]$$

Where J: flux value ($\mu g/h^{-1}cm^{-2}$); dQ/dt: Slope value from steady state curve; A: diffusion area; Cp: Coefficient permeability.

Stability of the salmon calcitonin NLCs-based emulgel

The stability study of the salmon calcitonin NLC-based emulgels was conducted following the stability study guidelines developed by ICH (2003) for finished products intended for refrigerated storage temperatures. The stability study conditions were 5 ± 3°C for the long-term storage conditions, as well as 25 ± 2°C and 60 ± 5% humidity for the accelerated storage conditions. Then, the salmon calcitonin content was measured at the 1st, 2nd, 3rd, and 6th months.

Chromatographic conditions for determination of salmon calcitonin content

The salmon calcitonin was analyzed by an HPLC instrument (Shimadzu, Japan) with a UV detector set at 215 nm. The separation was performed using a Zorbax Eclipse Plus C18 column (250 × 4.6 mm, 5 µm particles), and the mobile phase composition was water-acetonitrile (65:35, v/v) at an isocratic flow rate of 0.1 mL/min (Chen et al., 2013; Martenka, 2018; Tas et al., 2012).

Statistical analysis

In this study, all the result was presented as mean ± standard deviation. Statistical analysis to determine significant differences was performed using the t-test in Microsoft Excel 2022, with a probability value ($p<0.05$).

RESULTS AND DISCUSSION

Preparation of the salmon calcitonin-loaded NLCs

The salmon calcitonin-loaded NLCs were prepared by the homogenization method with ultrasonication. This method was applied because of its suitability for thermosensitive substances. Besides the selection of the manufacturing method, the excipients used in the formulation were also carefully selected. Acetic acid solution 0.05 M (pH 3.3) and acetate buffer (pH 5) were chosen due to their stability in maintaining salmon calcitonin in solution form (Martenka, 2018). Sodium cholate was chosen because its high hydrophilic-lipophilic balance (HLB 18) can encapsu-

late hydrophilic drugs, such as salmon calcitonin, more efficiently than low HLB surfactants (Manosroi et al., 2011; Zhang et al., 2017).

The surfactant and total lipid concentrations selected in the NLC vesicle preparations were based on our previous findings. We found that a formulation with 5% surfactant concentration and 75:25 solid lipid:liquid lipid ratio was superior given their characteristics such as spherical vesicles, particle size less than 200 nm, polydispersity index close to 0, zeta potential smaller than -30 mV, and high entrapment efficiency of salmon calcitonin. The concentration of salmon calcitonin in the first formulation was based on Garcia-Fuentes et al. (2005) study, which encapsulated salmon calcitonin into solid triglycerides nanostructured formulation at that concentration. Then, we varied the concentration of salmon calcitonin to observe its effect on vesicle characterization and *in vitro* penetration.

Characterization of the salmon calcitonin-loaded NLCs

The physicochemical characteristics of the salmon calcitonin-loaded NLCs are displayed in Table 3.

Particle size and zeta potential

The particle size, polydispersity index, and zeta potential of nanoparticles are determined using a dynamic light scattering method, which measures the Brownian motion of particles. Particle size and polydispersity index have a very close relationship. The polydispersity index has a value of 0 to 1, with a low PDI value (<0.5), indicating that the vesicles have a homogeneous dispersion (Danaei et al., 2018; Martihandini et al., 2021).

The result showed that the particle size of the four formulas was smaller than 400 nm. As shown in Table 3, the order of the NLCs particle size from the smallest was F2<F1<F3<F4, with polydispersity index ranging from 0.1-0.3. These results indicated that increasing the salmon calcitonin content in the formulation caused an increase in the size of the primary emulsion and further increased the size and polydispersity index of the final nanoparticles. Suhaimi et al. (2015) also provided the same statement that higher drug concentrations increased the particle size of NLC. On the other hand, the polydispersity index of nanoparticles could be influenced by the concentration of unabsorbed active substance molecules in the system, thus affecting the particle surface and the reading of unabsorbed drug molecules become polydisperse (Azmi et al. 2020).

Zeta potential results of the NLCs are shown in Table 3. Zeta potential has a significant role in describing the stability of a dispersion system, with a value of more negative than -30 mV or more positive than +30 mV can be said as a stable dispersion system. All formulas were detected to be negatively charged, with the stable formulas F1 and F2 (-32.43 mV and -34.67 mV). Meanwhile, formulas F3 and F4 produced values less negative than -30 mV. It may be influenced by increasing the salmon calcitonin as a cationic peptide that carries a positive charge at low pH (isoelectric point 10.4), it would affect the zeta potential by neutralizing the negative charge on the surfactant (sodium cholate). This reduced the zeta potential value from the acceptance range (±30 mV) (Sikora et al., 2015).

Morphology of NLC vesicles

The morphology analysis of salmon calcitonin-loaded NLCs is shown in Fig. 1, where the salmon calcitonin-loaded NLC vesicles had a particle size of approximately 100 nm. This result is also consistently identical to the particle size determined by the Zeta sizer, which showed F1 and F2 particle sizes around 100 nm. Furthermore, the transmission electron micrographs image also confirmed the shape of morphology F1 and F2 were spherical with smooth surfaces, and W/O emulsion granules filled the vesicles.

Table 3. Characteristic of salmon calcitonin-loaded NLC.

Formula NLC	Partikel size Z-average (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)
F1	169.13 ± 4.84	0.17 ± 0.04	-32.43 ± 0.50	97.00 ± 0.04
F2	135.60 ± 1.97	0.1 ± 0.001	-34.67 ± 1.72	99.62 ± 0.15
F3	258.17 ± 12.08	0.26 ± 0.04	-20.37 ± 1.35	97.50 ± 0.08
F4	360.33 + 20.8	0.34 ± 0.15	-19.8 ± 0.72	96.67 ± 0.47

All the data are expressed as mean ± SD (n = 3).

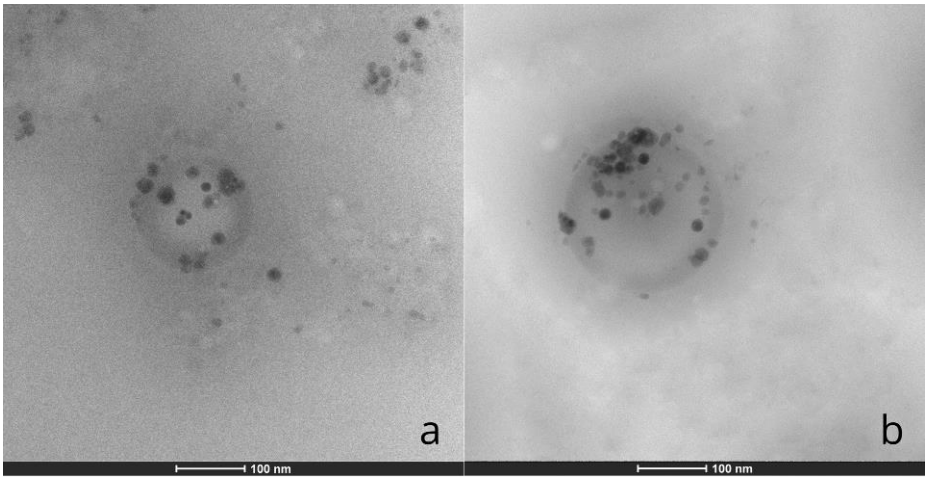

Figure 1. Morphological observations of NLC salmon calcitonin formula.

Transmission electron micrograph (TEM) result of salmon calcitonin loaded nanostructured lipid carrier formula. **(a)** NLC salmon calcitonin F1 with 70,000-95,000x magnification. **(b)** NLC salmon calcitonin F2 with 70,000-95,000x magnification.

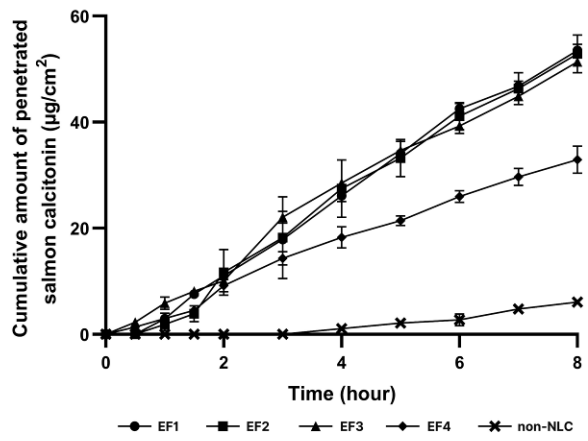

Figure 2. The cumulative amount of salmon calcitonin-loaded emulgel.

In vitro assay of salmon calcitonin emulgel was performed using a Franz diffusion apparatus for 8 hours. Each point is presented as mean ± SD with two repetitions. T-test analysis for the cumulative parameters of the diffusion test shows that statistically significant differences exist in all NLC-based emulgel formulas (EF1, EF2, EF3 and EF4) against the non-NLC emulgel. ($p<0.05$; 0.0002 EF1, 0.0003 EF2, 0.0001 EF3, and 0.004 EF4).

Entrapment efficiency

The entrapment efficiency of the salmon calcitonin-loaded NLC system was conducted using the indirect method. The indirect method was chosen because it was suitable for water-soluble active substances, such as salmon calcitonin, in an acetic acid solution (Martenka, 2018). The salmon calcitonin-loaded NLC entrapment efficiency of all formulas exceeded 95%. Table 3 showed that the salmon calcitonin-loaded NLC formula with the greatest absorption efficiency was F2>F3>F1>F4. These results showed that the concentration of the active substance in the system could affect the entrapment efficiency. This may be due to the low concentration of salmon calcitonin in the formulas (0.02-0.08%) compared to the vesicle in the formulation. In addition, the entrapment efficiency of salmon calcitonin into lipid nanoparticles was also due to the electrical attraction between salmon calcitonin and surfactant (sodium cholate). Sodium cholate is a bile salt compound that acts as an anionic surfactant that forms micelles in the formula. Salmon calcitonin, which had a positive charge value (pI 10.4) during the process, tended to be adsorbed on sodium cholate micelles, which had a negative charge. This caused electrostatic attraction resulting in salmon calcitonin trapped in the system becoming larger and preventing SCT from leaking in the inner water phase (Chen et al., 2013).

In vitro penetration study

The penetration study of salmon calcitonin NLCs-based emulgels is shown in Fig. 2. The formulas with the highest to lowest cumulative values were EF2>EF1>EF3>EF4, with values of 53.55; 52.85; 51.37; and 24.96 µg/cm², respectively. The cumulative amount of penetrated salmon calcitonin NLC-based emulgel EF1-EF4 was significantly ($p<0.05$) higher than the non-NLC emulgel formula with a value of

Table 4. Penetration flux, coefficient permeability, lag time, and enhancement ratio of salmon calcitonin NLCs-loaded emulgel.

Formula emulgel NLC	Cumulative amount (µg/cm²)	Flux (µg/cm².h)	Kp x 10^{-2} (cm/h)	Lag time (h)	Enhancement ratio
EF1	52.85	6.39 ± 0.41	6.39 ± 0.41	0	5.09
EF2	53.55	6.75 ± 0.40	6.75 ± 0.40	0	5.38
EF3	51.37	5.59 ± 0.12	5.59 ± 0.12	0	4.45
EF4	24.96	3.75 ± 1.16	3.75 ± 1.16	0.442	2.99
Non-NLC	6.05	1.26 ± 0.09	1.26 ± 0.09	3.328	1.00

All the data are expressed as mean ± SD (n = 2). T-test analysis for the enhancement ratio NLC formula (F1, F2, F3, and F4) indicated a significant difference ($p<0.05$) compared to Non-NLC emulgel.

6.05 µg/cm². Those significant amounts of calcitonin salmon achieved could be due to the small particle size of each formula, where there is a correlation between small particle size and the ability of particles to penetrate. As the particle size gets smaller, the cumulative penetration of salmon calcitonin into the stratum corneum gets higher (Hassan et al., 2022). NLC particles with a size ≤300 nm could penetrate the stratum corneum to deeper skin layers, while vesicles ≥600 nm remained in the stratum corneum (Martihandini et al., 2021). In addition to making the particle size smaller, the liquid lipid content also provided an occlusive effect on the skin so that it could increase the hydration of the stratum corneum and affect the percutaneous absorption (Pardeike et al., 2009; Teeranachaideekul et al., 2022). Besides particle size, the high entrapment efficiency may also contribute to the high cumulative result (Manosroi et al., 2012).

In previous research performed by Manosroi et al. (2013), the penetration value of salmon calcitonin using cell-penetrating peptide (CPP) carrier had a greater value, which was 58.36% at the 6th hour of diffusion, and in free salmon calcitonin had a value of 16.6%. These results show that the NLC that has been made has good potential in delivering salmon calcitonin transdermally, although the value produced is not as high as the results obtained using the CPP carrier.

The results of the *in vitro* penetration test can be seen in Table 4. The drug release rate or Flux (µg/cm²), permeability coefficient (Kp), enhancement ratio, and lag time or Tlag for each formula can be calculated. According to Fick's first law, the flux and lag time values are obtained from the equation at the steady state point in each formula. Compared to the non-NLC counterparts, salmon calcitonin NLC-based emulgel had higher flux values. EF2 had the fastest drug release rate, followed by EF1, EF3, and EF4, while the non-NLC formula had the slowest release rate. These results showed a significant difference ($p<0.05$) between NLC formula and with non-NLC formula.

In calculating lag time, formulas with lag time values are only in formula EF4 and non-NLC, with formula EF4 having a faster lag time than the non-NLC formula. This could be due to the larger particle size in NLC F4 (>300 nm) and the greater concentration of salmon calcitonin compared to EF1, EF2, and EF3. Meanwhile, in the formula of non-NLC salmon calcitonin emulgel, the slow lag time can be due to the properties of calcitonin as a hydrophilic and large molecular weight compound. The result indicates that the NLC system can help to reduce the time to penetrate the stratum corneum, and emulgel non-NLC has more time to penetrate and reach a steady state.

The effect of the NLC on the *in vitro* penetration could be expressed by the enhancement ratio. The salmon calcitonin formula in the NLC system showed higher flux and partition coefficient by three to five times than calcitonin salmon formula without NLC system. Therefore, the NLC-based emulgel had fivefold higher calcitonin penetration than the non-NLC emulgel. The high enhancement of salmon calcitonin NLCs-based emulgel penetration could be caused by NLC ability to create a monolayer on the epidermis and create an occlusive effect that increased the permeability of the compound (Arunprasert et al., 2022).

Stability study

Based on ICH 2003 guidelines on stability testing of finished products for refrigerated storage, the finished salmon calcitonin NLC emulgel was stored at 5 ± 3°C and 25 ± 2°C/RH 60 ± 5%. The stability test was observed for 6 months, with sampling intervals of sample data at months 0, 1, 2, 3, and 6. The stability test results of salmon calcitonin NLCs-based emulgels during the storage period can be seen in Fig. 3. The salmon calcitonin levels of all formulas during the

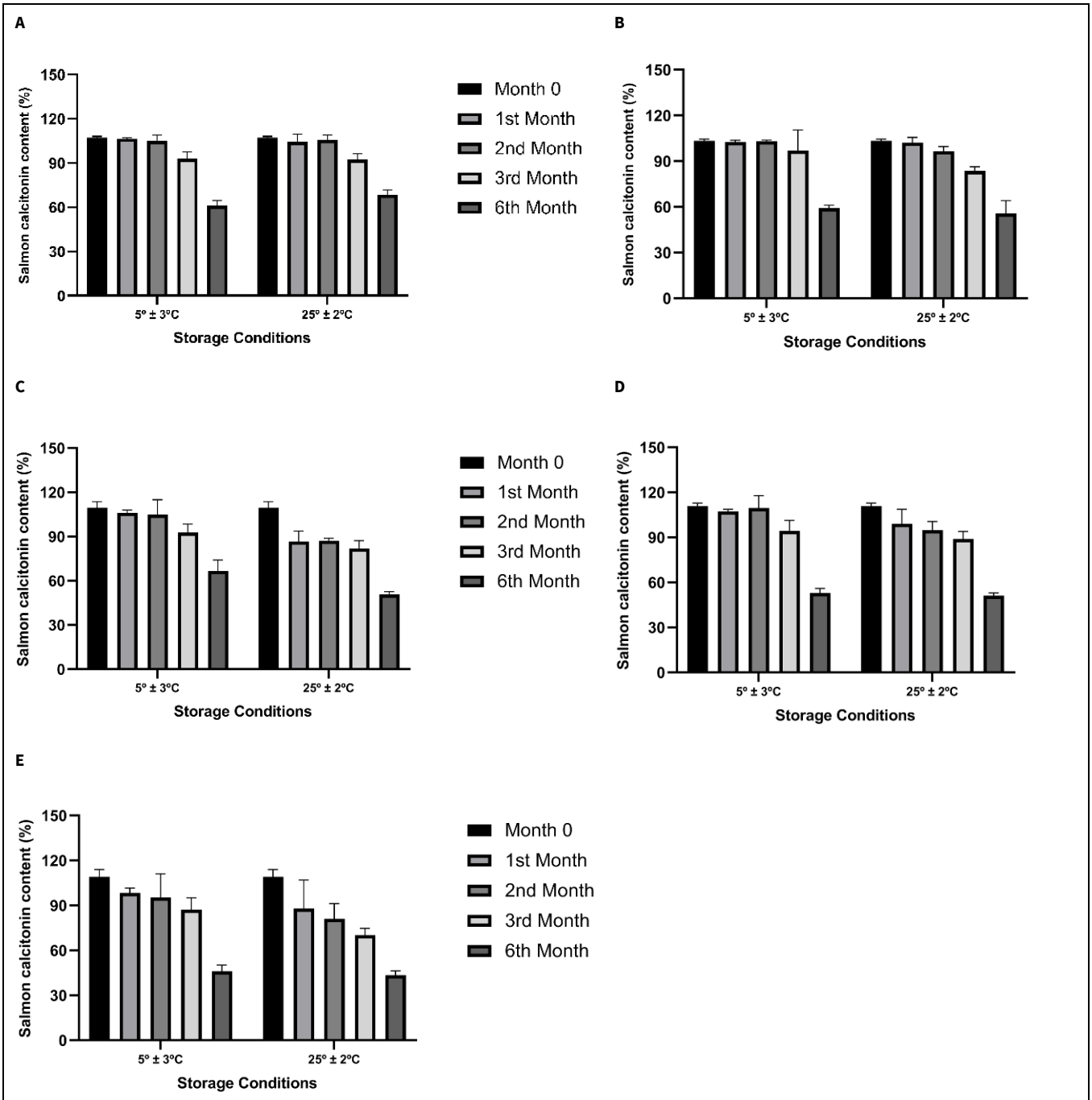

Figure 3. The stability test result of salmon calcitonin emulgel.

(A) NLC salmon calcitonin emulgel F1; **(B)** NLC salmon calcitonin emulgel F2; **(C)** NLC salmon calcitonin emulgel F3; **(D)** NLC salmon calcitonin emulgel F4; **(E)** NLC salmon calcitonin emulgel Non-NLC. Each bar is presented as mean ± SD (n = 3). Statistically significant differences were tested using T-Test analysis ($P<0.05$) between month 0 vs. month 6 in every sample. Statistically significant differences were also found in the month 6 salmon calcitonin concentrations of all formulas vs. non-NLC emulgel.

stability test had different values. In the first three months, the salmon calcitonin levels were still in the acceptance range (80-120%), respectively. However, in the sixth-month test of the NLC emulgel formula at 5 ± 3°C, the salmon calcitonin content decreased to 46.09-66.73%, whereas at 25 ± 2°C the salmon calcitonin content decreased to 50.65-68.59%. In the Non-NLC formula, salmon calcitonin concentration decreased to 46.09% at 5 ± 3°C and 43.45% at 25 ± 2°C. These results indicate that salmon calcitonin was a thermolabile compound with the best storage at a temperature 5 ± 3°C.

The concentration of salmon calcitonin on emulgel formula during shelf life (month 0 *vs.* month 6 showed significantly different ($p<0.05$). Another factor contributing to the decrease in salmon calcitonin

concentration during shelf life is the degradation effect caused by the pH of the emulgel. The pH of the emulgel is 5.5, which may cause an alkaline atmosphere beyond the stability limit of salmon calcitonin (3–4), even though the pH is within the skin acceptance range (4.5-6.5). This may have an impact on the stability of salmon calcitonin by causing it to degrade via the base-catalyzed dimerization pathway (Chang et al., 2003).

CONCLUSION

In this study, the calcitonin-loaded NLCs were successfully produced using a double emulsion-evaporation method with an entrapment efficiency of 99.6%. The satisfied calcitonin-loaded NLC was obtained from the formulation of the 75:1 ratio of total lipid to the drug (F2) with the appropriate particle characteristics for skin permeation enhancement. Therefore, the calcitonin penetration was fivefold enhanced by NLC based-emulgel compared to the non-NLC emulgel. Furthermore, the stability study indicated that the calcitonin-loaded NLCs were stable during three months at 5 ± 3°C, whereas the calcitonin concentration gradually decreased to 60% for six months. Consequently, further research is needed to improve its stability to embody its potential as transdermal delivery for calcitonin.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Zahrotunisa	Surini S
Concepts or ideas		x
Design	x	x
Definition of intellectual content		x
Literature search	x	x
Experimental studies	x	
Data acquisition	x	
Data analysis	x	x
Statistical analysis	x	
Manuscript preparation	x	x
Manuscript editing	x	x
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