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Research Article

EFFECT OF TRANSFERSOME FORMULATION ON THE STABILITY AND ANTIOXIDANT ACTIVITY OF N-ACETYLCYSTEINE IN ANTI-AGING CREAM

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ABSTRACT

Objective: N-acetylcysteine is an antioxidant with thiol/sulfhydryl groups and is currently being developed as an active ingredient in anti-aging creams. The study's aim was to compare the stability and antioxidant activity of N-acetylcysteine in anti-aging creams formulated with and without a transfersome carrier system.

Methods: Stability was assessed by performing cycling, centrifugal, and accelerated stability tests. In addition, antioxidant activity was measured by the DPPH method, and *in vitro* penetration was measured using Franz diffusion cells. The analysis of N-acetylcysteine was performed using high-performance liquid chromatography with ultraviolet–visible detection at a wavelength of 214 nm and a flow rate of 1.0 mL/min, injection volume of 5 µL, and a mobile phase of phosphate buffer pH 3.0.

Results: The N-acetylcysteine transfersome and non-transfersome cream preparations did not change color or show phase separation during the cycling and centrifugal tests. The N-acetylcysteine in the transfersome and non-transfersome cream preparations had strong antioxidant activity, with half-maximal inhibitory concentrations of 26.90 µg/mL and 38.63 µg/mL, respectively. The *in vitro* penetration test using Franz diffusion cells showed that the cumulative amount of penetrated N-acetylcysteine was 7355.13 $\mu g/cm^2$ (flux of 845.67 $\mu g/cm^2 \cdot h$) in the transfersome cream and 4677.61 $\mu g/cm^2$ (flux of 533.33 $\mu g/cm^2 \cdot h$) in the non-transfersome cream.

Conclusion: The *in vitro* penetration test results showed that the transfersome formulations in creams were able to increase the cumulative amount and flux of penetrated N-acetylcysteine in anti-aging cream preparations relative to those not formulated with transfersome.

Keywords: N-acetylcysteine, Transfersome, Anti-aging, Stability, Antioxidant, High-performance liquid chromatography.

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INTRODUCTION

Signs of aging can be partly prevented and slowed using various methods, one of which is the application of cosmetics. Cosmetics that are currently widely used to mask or slow signs of aging of the skin are called anti-aging cosmetics. Anti-aging products on the market are available in various dosage forms, one of which is a cream. Creams are generally preferred as topical dosage forms because cream is easily applied and easily removed from the skin [1]. Anti-aging cream products available on the market generally use an antioxidant as the active ingredient. Antioxidants are simple molecules that can prevent cell damage caused by oxidation stimulated by other molecules [2]. An antioxidant that has recently been developed as an active ingredient in anti-aging creams is N-acetylcysteine.

N-acetylcysteine is an antioxidant derived from the amino acid L-cysteine. N-acetylcysteine contains thiol/sulfhydryl (R-SH/-SH) groups, which are antioxidants [3]. N-acetylcysteine can prevent and/or inhibit the oxidative process as measured by different biomarkers of oxidative stress [4]. N-acetylcysteine can act as a direct antioxidant and indirect antioxidant. The mechanism of direct antioxidation involves a free thiol group that can interact directly with electrons from reactive oxygen species. In addition, N-acetylcysteine also can act as an indirect antioxidant because it can undergo deacetylation to cysteine, which is an intracellular reduced glutathione precursor that can increase glutathione levels in the body [3,4].

N-acetylcysteine is not stable under oxidative conditions because it is easily oxidized when formulated. Stabilization strategies are needed to maintain the stability of N-acetylcysteine formulations for use as antioxidative anti-aging creams. One strategy to maintain the stability of N-acetylcysteine is to formulate it with a transfersome carrying system [5]. The study's aim was to test the effects of transfersome systems on the stability and antioxidant activity of N-acetylcysteine in anti-aging formulations.

MATERIALS AND METHODS

Instrumentation

The following instrumentation was used: High-performance liquid chromatography (HPLC) (Model LC-20AT; Shimadzu, Japan); rotary vacuum evaporator (Buchi, Switzerland); ultraviolet (UV)–visible spectrophotometer (Shimadzu UV-1601 and Jasco Climate Chamber; Nuve Sanayi Malzemeleri Imalat ve Tic. A.S., Turki); vortex mixer (Thermo Scientific, America); centrifuge (Hettich Centrifugen EBA 200); microcentrifuge (Thermo Fisher Scientific); sonicator (Branson 3200); particle size analyzer (Malvern Zetasizer, Inggris); oven (Memmert); refrigerator (GEA, Germany); and Franz diffusion cell.

Materials

N-acetylcysteine standard (Daebong LS Co. LTD., Korea Selatan); phosphatidylcholine (Merck, Germany); Tween 80 (Merck); potassium dihydrogen phosphate (Merck, Germany); phosphoric acid (Merck); sodium hydroxide (Merck); sodium metabisulfite (Merck); dichloromethane p.a (Merck); distilled water (Ikapharmindo Putramas); and methanol (Merck) were used.

Preparation of mobile phase potassium dihydrogen phosphate pH 3.0

A 6.8 g amount of potassium dihydrogen phosphate was dissolved in 1000 mL of distilled water, adjusted with phosphoric acid to a pH 3.0, filtered, and degassed.

Preparation of sodium metabisulfite solution

A 0.25 g amount of sodium metabisulfite was dissolved in 500 mL of distilled water, freshly prepared [6].

Preparation of N-acetylcysteine 400 μg/mL standard solution

A 100 mg amount of N-acetylcysteine standard was accurately weighed and placed in a 10 mL volumetric flask, dissolved, and made up to volume with sodium metabisulfite solution to give a standard stock solution of 10.000 μg/mL. The standard stock solution was then diluted to a concentration of 1000 μg/mL and diluted again to give a concentration of 400 μg/mL [6].

Determination of optimum analysis conditions

The following general chromatographic conditions were used to analyze N-acetylcysteine: Shimpack C18 column (5 μm, 4.6×250 mm), sodium metabisulfite solution as solvent, potassium dihydrogen phosphate solution pH 3.0 as mobile phase, injection volume of 5 μL, and detection wavelength of 214 nm. The optimum analysis conditions were determined by varying the flow rate at 0.8, 1.0, and 1.2 mL/min. The standard N-acetylcysteine 400 μg/mL test solution was injected (≤5 μL) into the HPLC. The selected optimized chromatographic conditions were those that gave the highest peak area, a relatively short retention time (tR), the largest number of theoretical plates (N), the smallest height equivalent to a theoretical plate (HETP; the best potential separation efficiency), and the smallest tailing factor (Tf).

System suitability test

A 5 μL aliquot of the standard solution of N-acetylcysteine (400 μg/mL) was injected 6 times into the HPLC under the optimum analysis conditions. The peak area, tR, N, HETP value, resolution (R), and Tf were determined and averaged. The results were obtained at the optimum wavelength and analysis conditions. The results fulfilled the system suitability test requirements of coefficient of variation (CV) <2%.

Validation of HPLC method

Linearity, limit of detection (LOD), and limit of quantitation (LOQ)

A standard solution of N-acetylcysteine (1000 μg/mL) was diluted to give 100, 200, 300, 400, 500, and 600 μg/mL working solutions. Then, 5 μL of a working solution was injected into the HPLC under the optimum analysis conditions [6]. A calibration curve was created from the peak area (y) versus the concentration of analyte (x). A linear regression equation and the correlation coefficient for the fitted curve were determined. Based on the calibration curve obtained, the LOD and LOQ were calculated. The results fulfilled linearity requirements, with a correlation coefficient r>0.9990.

Selectivity test

A blank solution (cream matrix) and standard solution of N-acetylcysteine 400 μg/mL were prepared. A 5 μL aliquot of the blank and standard solutions were separately injected into the HPLC under the optimum analysis conditions. The results of the blank and standard selectivity test chromatograms were compared, and the chromatograms were evaluated to determine if there was interference or the appearance of another peak around the tR of N-acetylcysteine. Selectivity was assessed to be good if there was no interference and no other peaks around the N-acetylcysteine tR.

Accuracy and precision

Accuracy and precision were determined using a simulation method (spiked-placebo recovery), which involved adding a number of pure material analytes to the carrier material for pharmaceutical preparations (placebo). Then, the mixture was analyzed under the optimum analysis conditions, and the results were compared with the analyte levels added (actual levels). The concentration used in the test of accuracy and precision of N-acetylcysteine was 50% (200 μg/mL), 100% (400 μg/mL), and 150% (600 μg/mL) [7]. The 50% concentration was made by mixing 20 mg of N-acetylcysteine standard into a 500 mg cream matrix. The 100% concentration was made by mixing 40 mg of N-acetylcysteine standard into a 500 mg cream matrix. The concentration of 150% was made by mixing 60 mg of N-acetylcysteine standard into a 500 mg cream matrix. For each of these concentrations, the sample was accurately weighed and dissolved in 10 mL of sodium metabisulfite solution in a volumetric flask. The solution of each concentration was sonicated for 30 min and vortex mixed for 5 min, then centrifuged at 4000 rpm for 15 min. The supernatants from each concentration were filtered through 0.45 μm nylon syringe filters, and then 5 μL were injected into the HPLC under the optimum analysis conditions. The 50% and 150% concentrations were analyzed with triplicate injections, and the 100% concentration was analyzed with 6 injections.

Each concentration was injected into the HPLC under the optimum analysis conditions and the peak area of N-acetylcysteine was integrated and recorded. The percent recovery (%UPK) was calculated by comparing the measured concentration with the actual concentration. Acceptable accuracy was a %UPK ranging from 98% to 102%. Precision was determined as the relative standard deviation or CV. Acceptable precision was a CV <2% [8].

Transfersome formulation

Transfersome formulations were prepared by a thin-layer hydration method. The transfersome formulations are presented in Table 1. Transfersome was formulated by mixing phosphatidylcholine and Tween 80 and dissolving them in dichloromethane. The solution was then placed in a round-bottom flask and evaporated on a rotary vacuum evaporator for 60 min at a temperature of 40°C and 50–150 rpm. Nitrogen gas was passed over the thin residual layer for 2 min, and the flask was then stored in a refrigerator for 24 h in a closed state. The thin layer was hydrated with a phosphate buffer solution (pH 7.4) containing N-acetylcysteine in a rotary vacuum evaporator at a temperature of 30°C and rotation speed of 50–150 rpm for 60 min with glass beads to remove the thin layer on the round flask wall. Ultrasonication was performed for 10 min to reduce the particle size of the solids [7,9-11].

Transfersome optimization

Particle size and polydispersity index

Determination of the particle size and polydispersity index of the transfersome formulations were determined by the dynamic light scattering method using a particle size analyzer at 25°C. One drop of sample from each transfersome formulation was dispersed in 10.0 mL of distilled water and then tested for particle size.

Entrapment efficiency

Entrapment efficiency was measured by calculating the total and released N-acetylcysteine concentrations in a transfersome suspension. The released concentration of N-acetylcysteine measurement was obtained by dissolving 1 mL of transfersome suspension in 10 mL of sodium metabisulfite solution and then centrifuging at 13,000 rpm for 2 hat 4°C. The supernatant was filtered and injected into the HPLC.

Transfersome and non-transfersome cream preparations

The optimum transfersome suspension was freeze-dried. A dry transfersome suspension equivalent to 5% N-acetylcysteine was added gradually into the cream base and then mixed by using a homogenizer at 500 rpm. In a non-transfersome cream, N-acetylcysteine was added at 5% into the cream base and mixed using a homogenizer at 500 rpm.

Table 1: Mobile phase selection

Material	F1 (g) (90:10)	F2 (g) (85:15)	F3 (g) (75:25)
N-acetylcysteine	1.5	1.5	1.5
Phosphatidylcholine	1.35	1.275	1.125
Tween 80	0.15	0.225	0.375
Phosphate buffer solution pH 7.4	12	12	12
Total	15	15	15

Stability tests

Cycling test

The two cream samples were stored at 4°C for 24 h and then transferred to an oven at 40°C±2°C for 24 h, which constituted one cycle. Six more cycles were performed over 12 days. The physical conditions of creams, such as organoleptic characteristics (discoloration, odor, and homogeneity), were observed before and after the experiment.

Centrifugal test

Both cream samples were inserted into the centrifugation tube and then centrifuged at 3750 rpm for 5 h. After centrifugation, the sample was observed to determine if phase separation between the water and oil phases occurred.

Accelerated stability testing

Both cream samples were stored in a climate chamber with storage conditions of 40°C±2°C and relative humidity of 70%±5% RH for 3 months. Sampling was carried out at 0, 1, 2, and 3 months and analyzed using HPLC under optimum analysis conditions.

Approximately 80 mg of each stability test sample was accurately weighed and dissolved in 10 mL with sodium metabisulfite solution in a volumetric flask. The sample solution was sonicated for 30 min and vortex mixed for 5 min, then centrifuged at 4000 rpm for 15 min. The supernatant of each sample was filtered and injected into the HPLC under the optimum analysis conditions. This procedure was performed in triplicate for each sample.

Antioxidant activity test using DPPH assay

Approximately 250 mg of each cream sample (containing 12.5 mg N-acetylcysteine) was weighed accurately and placed into a 25.0 mL volumetric flask, dissolved, and made up to volume with methanol. The sample solution was then sonicated for 10 min and centrifuged at 4000 rpm for 10 min. The supernatant of each sample was filtered, and a 500 µg/mL sample stock solution was obtained. The sample stock solution was then diluted to a concentration of 100 µg/mL and diluted again to obtain sample concentrations of 1, 5, 10, 15, 20, and 30 µg/mL. A 3.0 mL aliquot of diphenyl picrylhydrazyl (DPPH) was added to 1.0 mL of each sample solution. Methanol (1.0 mL) and DPPH 50 µg/mL (3.0 mL) solutions were used as a blank. The mixture was shaken for 20 s and incubated in a dark room at room temperature (27°C±2°C) for 30 min.

The antioxidant activity was determined by measuring the absorbance of each sample by UV–visible spectrophotometry at an optimum wavelength of 515 nm. The result obtained was used to calculate the inhibition percentage of DPPH by N-acetylcysteine in cream samples. The percentage of DPPH radical inhibition was calculated by comparing the sample and blank results.

Franz diffusion cells *in vitro* penetration test

The membrane used was the abdomen skin of 2–3-month-old female Sprague-Dawley rats weighing 150–200 g. In this study, all methods for sacrificing the animals had been approved through an ethical approval certificate from the Health Research Ethics Committee, Faculty of Medicine, University of Indonesia No.184/UN2.F1/ETIK/PPM.00.02/2019. *In vitro* penetration tests were performed using Franz diffusion cells with a diffusion area of 1.77 cm^2 and a compartment volume of 15.0 mL. The receptor compartment was filled with ≤15.0 mL phosphate-buffered solution pH 7.4 and the temperature was maintained using a water jacket at 37°C±0.5°C and stirred with a magnetic stirrer at a speed of 200 rpm. The abdominal skin was shaved and cleaned of fat and then placed between the donor and receptor compartments with the position of the stratum corneum facing up. The cream samples tested in this study were 5% N-acetylcysteine transfersome cream and 5% N-acetylcysteine non-transfersome cream. Each cream sample (1 g) was applied to the skin in the donor compartment. Sample solution (1 mL) was taken from the receptor compartment using a syringe at 10, 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min and immediately refilled with the same amount of phosphate buffer pH 7.4. The sample solution was inserted into a vial, and its concentration was measured by HPLC under the optimum analysis conditions.

RESULTS AND DISCUSSION

Determination of optimum analysis conditions

The results of the analysis method optimization are presented in Table 2.

The flow rate chosen in this study was 1.0 mL/min because it gave better results than those at the flow rate of 0.8 mL/min and 1.2 mL/min. The flow rate of 1.0 mL/min had a large peak area of 1,130,622 µV/s, the retention time of 8.718 min was relatively early, and the Tf of 1.446 was the smallest of the tested conditions. The flow rate of 0.8 mL/min was not chosen even though it had a larger peak area than that at 1.0 mL/min because the retention time of 11.236 min was too long and the Tf was higher.

System suitability test

The system suitability test results are presented in Table 3.

The system suitability test was performed first before analysis under the optimum analysis conditions. After injecting the 400 µg/mL standard

Table 2: Peak area, retention time, tailing factor, column efficiency, and theoretical plates from N-acetylcysteine chromatograms at two eluent flow rates

Flow rate (mL/min)	Peak area (µV/s)	Retention time (min)	Tailing factor	HETP (cm)	Theoretical plates (N)
0.8	1,470,303	11.236	1.516	40.187	3733
1.0	1,130,622	8.718	1.446	41.683	3599
1.2	9,59,846	7.436	1.454	44.133	3399

Table 3: System suitability test results of N-acetylcysteine

Concentration (µg/mL)	Peak area (µV/s)	Retention time (min)	Tailing factor	Height equivalent to a theoretical plate (cm)	Theoretical plates (N)
	1,144,078	8.980	1.469	41.758	3592
	1,147,670	8.979	1.500	42.551	3525
400	1,150,640	8.718	1.479	41.826	3586
	1,141,042	8.642	1.498	41.558	3690
	1,155,160	8.686	1.495	40.543	3700
	1,149,613	8.620	1.454	41.596	3606
Average	1,148,033.83	8.771	1.483	41.639	3617
Standard deviation	4994.6942	0.1652	0.0185	0.6466	66.9141
Coefficient of variation (%)	0.44	1.88	1.25	1.55	1.85

solution of N-acetylcysteine 6 times, the coefficients of variation were 0.44% for peak area, 1.88% for retention time, 1.25% for Tf, 1.55% for HETP, and 1.85% for the number of theoretical plates (N). The CV did not exceed 2%, as required [8].

Validation method

Linearity, LOD, and LOQ

The calibration curve data, LOD, and LOQ of N-acetylcysteine are presented in Table 4.

Fig. 1 shows the calibration curve for N-acetylcysteine. The results obtained are excellent because they have a correlation coefficient close to 1 (r>0.9990) [8]. The LOD and LOQ values were sufficiently sensitive.

Selectivity test

The chromatogram from the blank solution did not show any interference or peaks of other compounds at the retention time of N-acetylcysteine, which supports the selectivity of the method for N-acetylcysteine [8].

Accuracy and precision

The accuracy and precision of the method are presented in Table 5.

The precision fulfilled the requirements by giving a CV of ≤2% [8]. The method used in this study fulfilled the criteria for accuracy and precision.

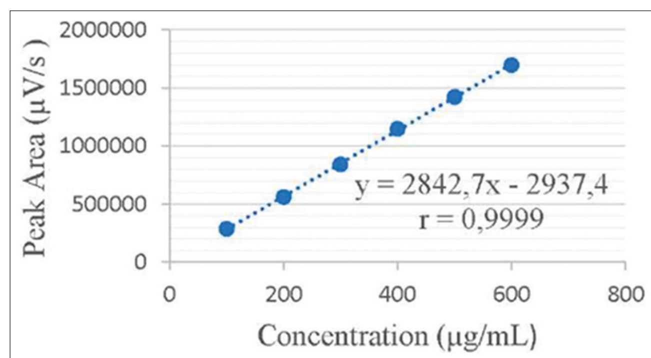

Fig. 1: Calibration curve of N-acetylcysteine

Transfersome formulation optimization

The transfersome formulation optimization results are presented in Table 6.

Particle size

The Z-average value shows the average particle size in the preparation. The particle sizes were good and were <200 nm [12]. The results show that higher concentrations of surfactant gave larger particle size [13].

Polydispersity index

The particle size distribution is reflected by the polydispersity index parameter and is used to show the particle size distribution in nanoparticle systems. The polydispersity index values ranged from 0 to 0.6. If the value is >0.6, the sample has a very wide size distribution; as the value approaches zero, the particle size distribution is increasingly homogeneous. The resulting values were good because they were <0.6 [12]. The results showed that the distribution of particle size in F1 was the most homogeneous. The polydispersity index of each formula increased with increasing particle size [14].

Entrapment efficiency

Determination of entrapment efficiency was performed to determine the amount of active substances that were absorbed by transfersome. The highest entrapment efficiency was 65.58% for F1. The entrapment efficiency of transfersome increased with increasing lipid concentration (phospholipid) and decreased with increasing surfactant concentration because lower surfactant concentrations (<15%, b/b) allowed all surfactant molecules to bind to the phospholipid bilayer layer. If the concentration exceeds a certain concentration, some surfactant molecules will cause an increase in the permeability of the vesicle membrane, which can reduce the efficiency of entrapment [15,16]. Phospholipids are the main components that form vesicles. The higher the phospholipid concentration used, the more vesicles are formed, so the absorption of active substances is also expected to be more optimal.

Based on the results of particle size and the calculation of entrapment efficiency, F1 was selected as the optimum formula for the cream preparations because it had the smallest particle size, with a Z-average of 57.76 nm, the smallest polydispersity index value of 0.282, and the largest entrapment efficiency of 65.58%.

Table 4: Calibration curve data, LOD, and LOQ of N-acetylcysteine

Concentration (μg/mL)	Peak area (μV/s)	yi=a+bx		S (y/x)	LOD (μg/mL)	LOQ (μg/mL)
100	284,997	281,332.60	13,427,827.36			
200	559,628	565,602.60	35,695,845.16			
300	842,068	849,872.60	60,911,781.16	9039.00	9.54	31.80
400	1,147,729	1,134,142.60	184,590,264.96			
500	1,420,344	1,418,412.60	3,730,305.96			
600	1,697,348	1,702,682.60	28,457,957.16			
n=6			∑=326,813,981.7			

LOD: Limit of detection, LOQ: Limit of quantitation

Table 5: Accuracy and precision data of N-acetylcysteine

Concentration (μg/mL)	Peak area (GiV/s)	Measured conc. (μg/mL)	Recovery (%)	Average (%)	Standard deviation	Coefficient of variation (%)
	570,152	201.60	100.80			
200	573,738	202.86	101.43	100.96	0.8214	0.41
	569,354	201.32	100.66			
	1,146,385	404.31	101.08			
	1,137,370	401.13	100.28			
400	1,140,504	402.24	100.56	100.88	1.5621	0.39
	1,149,383	405.36	101.34			
	1,146,786	404.45	101.11			
	1,144,255	403.56	100.89			
	1,733,954	611.00	101.83			
600	1,722,616	607.01	101.17	100.76	7.9127	1.31
	1,690,583	595.74	99.29			

Stability test

Cycling test

The purpose of the cycling test was to determine the physical changes in the two creams. After testing for six cycles between 4°C and 40°C±2°C, the results showed that neither cream showed a color change nor a phase separation. However, the smell of the non-transfersome cream had an unpleasant sulfurous odor. Both creams showed good and stable results because they did not show phase separation and remained homogeneous. However, the transfersome cream was more stable than the non-transfersome cream because it did not experience a change in odor.

Centrifugal test

The centrifugal test was performed to determine the stability of the cream after a very strong shaking after being centrifuged at a speed of 3750 rpm for 5 h. This speed is equivalent to the gravitational force experienced over 1 year. After testing, the two creams did not show any separation of the water phase and oil phase, which was probably because of the use of appropriate emulsifying agents. In addition, phase separation can occur during the stirring process, but an appropriate stirring speed was used, which prevented separation during testing. Based on these results, we concluded that the two creams could withstand the force of gravity for 1 year.

Accelerated stability testing

The accelerated stability testing results are shown in Table 7. The curve of accelerated stability testing results of N-acetylcysteine in transfersome cream and non-transfersome cream is shown in Fig. 2.

The accelerated stability test was performed to determine the stability of the two creams under conditions that can accelerate the change in stability at 40°C±2°C and relative humidity of 70% ±5% RH for 3 months. Storage of cream under accelerated conditions can result in a decrease in the level of N-acetylcysteine. This caused the remaining N-acetylcysteine levels in the transfersome creams after 3 months to be 82.92% and non-transfersome creams to be 48.47%.

Table 6: Transfersome optimization results

Formulation	Z-average (nm)	Polydispersity index (nm)	Entrapment efficiency (%)
F1	57.76	0.282	65.58
F2	62.62	0.349	62.19
F3	102.4	0.385	59.72

Table 7: Accelerated stability testing results

Sample	Average remaining level of N-acetylcysteine (%)	
	Transfersome cream	Non-transfersome cream
Month 0	99.67	99.69
Month 1	94.71	86.06
Month 2	85.31	52.97
Month 3	82.92	48.47

Decreased levels of N-acetylcysteine in transfersome cream and non-transfersome cream preparations can occur because of an oxidation reaction in N-acetylcysteine compounds. N-acetylcysteine is easily oxidized to the disulfide form, N,N-diacetylcysteine [17,18]. The easily oxidized group is the thiol/sulfhydryl (-SH) group. Decreasing levels in cream preparations can also be caused by other factors, such as the presence of water in the cream, the effect of temperature, and the effect of humidity under accelerated conditions [19].

Transfersome is a carrier system that is the development of liposomes. Transfersomes have almost the same properties as liposomes, but a transfersome has a deformability that can increase drug penetration through the skin [13]. Transfersome formulations in creams can increase the stability of N-acetylcysteine because transfersome vesicles can protect the active substance so that contact with oxidizing agents can be avoided. In non-transfersome creams, N-acetylcysteine compounds are not coated by a vesicle transfersome, so they are easily oxidized. This leads to lower N-acetylcysteine levels in non-transfersome creams than those in transfersome creams [19].

N-acetylcysteine that has been formulated in the form of transfersomes can show decreased levels, similar to those in non-transfersome creams. This can be because the entrapment efficiency in the optimum transfersome formulation used was 65.58%, so there is still some free N-acetylcysteine that can be oxidized as in a non-transfersome cream. In addition, decreased N-acetylcysteine levels can also be caused by damage to the transfersome vesicles due to the degradation of the lipid bilayer as temperature increases. Lipid degradation can also occur because of chemical degradation processes that can affect the stability of the phospholipid bilayer, such as hydrolysis and lipid oxidation. Degradation of the lipid bilayer can reduce the absorption efficiency of N-acetylcysteine so that more free N-acetylcysteine is oxidized [15].

Based on the average level of N-acetylcysteine remaining in the two cream samples, the shelf life of each cream was calculated using a zero-order stability test. Based on the calculated results, the shelf life of transfersome cream N-acetylcysteine was 3 months 10 days, and the shelf life of non-transfersome cream N-acetylcysteine was 1 month 2 days. Based on these results, we concluded that the transfersome formulation in the preparation of anti-aging cream N-acetylcysteine can increase the stability of N-acetylcysteine in anti-aging cream preparations.

Antioxidant activity test using DPPH assay

DPPH is a stable free radical compound that has one free electron on its nitrogen atom. This test is based on the principle that DPPH accepts hydrogen atoms (H) from antioxidant molecules, which results in a more stable DPPH-H reduced form. The purple color in the DPPH

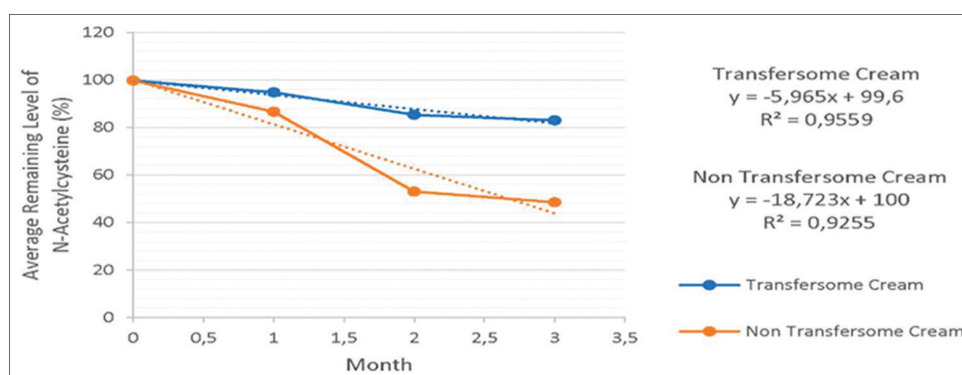

Fig. 2: The curve of accelerated stability testing results of N-acetylcysteine in transfersome cream and non-transfersome cream

solution will turn yellow as electrons are captured by DPPH, which indicates an increase in the reduced form of DPPH-H [20-23].

The maximum DPPH wavelength was 515 nm and was used to measure the antioxidant activity of the sample. The half-maximal inhibitory concentration (IC_{50}) of N-acetylcysteine was 26.90 µg/mL in transfersome cream and 38.63 µg/mL in non-transfersome cream. A compound is said to have very strong antioxidant activity if the IC_{50} is <10 µg/mL, strong if it is between 10 and 50 µg/mL, moderate if it is between 50 and 100 µg/mL, weak if it is between 100 and 250 µg/mL, and not active if it is >250 µg/mL [24]. Based on the results obtained, N-acetylcysteine in the transfersome and non-transfersome creams had strong antioxidant activity because the IC_{50} was between 10 and 50 µg/mL [24]. The IC_{50} value is the concentration of antioxidant compounds needed to inhibit 50% of existing DPPH activities. Smaller IC_{50} values have a stronger potential for antioxidant activity in these compounds. N-acetylcysteine is an antioxidant derived from the amino acid L-cysteine, in which the group that is an antioxidant is a thiol/sulfhydryl group (-SH) [3].

The DPPH method antioxidant activity test results of anti-aging cream N-acetylcysteine are shown in Table 8.

Franz diffusion cells *in vitro* penetration test

In vitro penetration tests have two main parameters: The cumulative amount of penetrated active substances (µg/cm²) and penetration rate (flux) [8,25,26]. Based on the results obtained after 8 h of sampling, the cumulative amount of N-acetylcysteine penetrated was greater in the transfersome cream (7355.13 µg/cm²) than in the non-transfersome cream (4677.61 µg/cm²). In addition, the flux values obtained from the slope taken from the linear curve at steady state were based on Fick's First Law. Flux values in the steady-state were higher for the transfersome cream preparations (845.67 µg/cm²·h) than for the non-transfersome N-acetylcysteine creams (533.33 µg/cm²·h). These results prove that the penetration rates of N-acetylcysteine were faster from the transfersome cream preparations than from the N-acetylcysteine non-transfersome cream.

Table 8: DPPH method antioxidant activity test results of anti-aging cream N-acetylcysteine

Sample	Linear regression	IC_{50} (µg/mL)
Transfersome cream N-acetylcysteine	y=0.5417x+35.43 r=0.9979	26.90
Non-transfersome cream N-acetylcysteine	y=0.4114x+34.109 r=0.9980	38.63

Table 9: DPPH method antioxidant activity results of anti-aging cream N-acetylcysteine

Sample	Cumulative amount of N-acetylcysteine penetrated (µg/cm²)	Flux value of N-acetylcysteine (µg/cm⁻²/h)
Transfersome cream N-acetylcysteine	7355.13	919.39
Non-transfersome cream N-acetylcysteine	4677.61	584.70

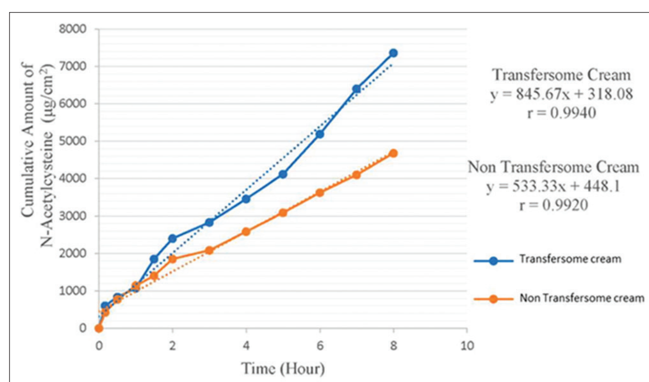

Fig. 3: The cumulative amount of N-acetylcysteine penetrated and flux from N-acetylcysteine transfersome and non-transfersome cream preparations

The results of the penetration tests performed on the transfersome creams and N-acetylcysteine non-transfersome creams showed a significant difference. Formulating N-acetylcysteine into a transfersome cream increased the cumulative amount and *in vitro* flux of N-acetylcysteine in anti-aging creams relative to those of the non-transfersome creams. In the transfersome creams, vesicular transfersomes act as penetration enhancers because the vesicles enter the stratum corneum and then modify the intracellular lipid lamellae. Transfersome vesicles facilitate penetration of N-acetylcysteine through the lipid bilayer membrane of the skin stratum corneum, which has pores smaller than the size of the drug itself. In addition, phospholipids have a high affinity for biological membranes, so incorporation of phospholipid bilayer vesicles with intracellular lipid layers onto the skin through a cream can contribute to increased permeability of transfersome [9,16,27].

The antioxidant activity results of anti-aging cream N-acetylcysteine are shown in Table 9. The cumulative amount of N-acetylcysteine penetrated and flux from N-acetylcysteine transfersome and non-transfersome cream preparations are shown in Fig. 3.

CONCLUSION

The optimum HPLC analysis conditions for analyzing N-acetylcysteine compounds in anti-aging creams were identified and found to be suitable for future analyses. The optimum transfersome formulation used in the anti-aging cream N-acetylcysteine preparations had an entrapment efficiency of 65.58%, a particle size of 57.76 nm, and a polydispersity index of 0.282. Based on the results of the stability test, N-acetylcysteine was more stable in transfersome cream than in non-transfersome cream. The average N-acetylcysteine content remaining after accelerated stability testing for 3 months was 82.92% with a shelf life of 3 months 10 days in the transfersome cream and was 48.47% with a shelf life of 1 month 2 days in the non-transfersome cream. In addition, physical stability was relatively better in the N-acetylcysteine transfersome creams than in the non-transfersome creams, as shown by the cycling test and centrifugal test results because there was no change in odor as observed for the non-transfersome creams. Future studies should attempt to identify substances that may be formed as a result of the degradation of N-acetylcysteine in anti-aging creams formulated using transfersomes.

CONFLICTS OF INTEREST

All authors have none to declare.

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