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Development and Evaluation of Sodium Ascorbyl Phosphate Nanoemulsion For Transcutaneous Delivery

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ABSTRACT:

Sodium ascorbyl phosphate (SAP) nanoemulsion has been developed using several methods with the aim of improving its skin permeation by transcutaneous delivery. SAP is a hydrophilic derivate of ascorbic acid with low permeability. Development of the formulation began with the utilization of glycerin as the solvent as well as co-surfactants, combined with the addition of amphiphilic molecules and lipophilic surfactants. Amphiphilic molecules and lipophilic surfactants formed complexes and emulsion, respectively, then underwent co-lyophilization prior to incorporating the lyophilizate to form nanoemulsion. Polyethylene glycol (PEG) 20000 and lecithin were used as the amphiphilic molecules, while Span 80 was employed as lipophilic surfactants. These methods aimed to improve the permeability of SAP, thus enhancing its permeation across the stratum corneum. The highest SAP solubility was achieved by adding 10% glycerin, which meant the glycerin could be used to increase the lipophilicity of SAP in the formulation. The amount of SAP partitioned into the oil phase was mixed by solid-in-oil dispersion (SOD) with PEG 20000, lecithin, and Span 80 was determined indirectly by measuring the concentration of SAP in the aqueous phase. The globule sizes of the SAP-PEG 20000 and SAP-lecithin nanoemulsions were 50–200 nm and > 500 nm for the SAP-Span 80 nanoemulsion. The amount of diffused SAP was varied in an *in vitro* permeation study using the Franz diffusion cell, snake skin (*Python R.*) and Spangler's as a membrane models. This study indicates that SAP could penetrate in to the skin by SOD method and its potential utility to improve the skin permeation of hydrophilic molecules.

KEYWORDS: Sodium ascorbyl phosphate; lyophilization; nanoemulsion; permeation; amphiphilic.

INTRODUCTION:

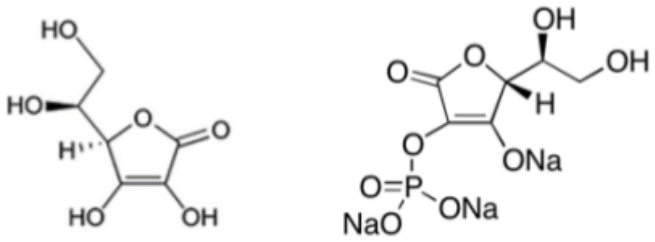
The system for delivering an active substance through the skin transcutaneously or transdermally is limited by the size, molecular weight, and molecular affinity of the skin layer composed of lipid components. Transcutaneous delivery of hydrophilic compound has been extensively studied in the last few years using protein as a model active ingredient. Such studies have reported that hydrophilic molecules are able to cross the lipid layer of the cutaneous membrane by the solid dispersion method using amphiphilic polymer to improve their dispersibility in oil[1-5].

Sodium ascorbyl phosphate or SAP (shown in figure 1) is an ascorbic acid derivative with very hydrophilic nature and more stable against oxidation. SAP will be converted into free ascorbic acid (shown in figure 1) through the enzymatic degradation process in the skin where it can acts as an antioxidant to induce collagen production in aged skin[6]. SAP, also known as L-ascorbic acid-2-monophosphate tri-sodium salt, has a solubility of 782 g/L in water at 20 °C. İt has a low permeability constant (log P of 10^{-4}), indicating its limited skin permeation in the stratum corneum (SC) layer [6,7].

This study aims to developed transcutaneous delivery of SAP as an antiwrinkle by increasing the oil dispersion using various techniques. The S/O dispersion of SAP was subsequently developed into a nanoemulsion formula having small oil droplet diameter to facilitate intercellular lipid permeation of the vesicle[4,5]. Increasing the SAP dispersion in oil at sub-nanometer sized is the point of this research, which was conducted by simply adding glycerin or developed through co-lyophilization process by combining it with amphiphilic compounds or a lipophilic surfactant. In the first method, glycerin was selected as a solvent mixture to dissolve SAP in virgin coconut oil (VCO) employed as the oil phase of nanoemulsion. The solubility of SAP in glycerin is 13.2%[6]. Furthermore, glycerin was also used as a co-surfactant for stabilizing nanoemulsion formulation. Meanwhile, soya lecithin and polyethylene glycol (PEG) was employed in the second method which the mixture of these compounds with SAP in water was solidified by co-lyophilization process. Subsequently, the co-lyophilizate was dispersed in VCO where the amphiphilic molecules were proposed, to provide a contact layers surrounding SAP particles in the oil phase. Soy bean lecithin is an apolar organic solvent, and natural bio-friendly molecules that can used to improve skin penetration to skin layers[8].

In the last method, lipophilic surfactant, i.e Span 80, was used to form an amphiphilic layer on SAP particles in oil following freeze drying process of an intermediate SAP water-in-oil nanoemulsion. The SAP solid dispersion in oil was hypothesized to prevent particle aggregation and minimize SAP diffusion into the aqueous phase in the final oil-in-water nanoemulsion[9-14]. The formation of SAP solid dispersion in-oil by co-lyophilization method developed in this research was addressed to eliminate the use of organic solvent applied by previous research.

Improvement of SAP in the oil phase has a great challenge because the tendency of SAP to diffuse to the water phase. It has been reported that the oil dispersion of hydrophilic compounds with lipophilic surfactant within transdermal or transcutaneous delivery in nanoformulation resulting good skin penetration[12,13,14]. Nanoemulsion has a small globules diameter and the delivery is expected to be able to keep a SAP molecules in the oil phase and then penetrate well to the stratum corneum (SC) layer.



Vitamin C **Sodium ascorbyl phosphate (SAP)**

Fig.1 Chemical Structure of Vitamin C and SAP

MATERIAL AND METHODS:

Chemicals and reagents:

The SAP was a gift from BASF (Ludwigshafen, Germany). The VCO was a product of the School of Life Sciences and Technology, ITB (Bandung, Indonesia). Polyethylene glycol (PEG) 20000 was obtained from Fluka, Singapore. Lipoid S100® was purchased from Landson (Indonesia). Tween 80, PEG 400, glycerin, proplene glycol, and ethanol were purchased from Bratachem (Indonesia). Methanol, acetonitrile, KH_2PO_4, and Na_2HPO_4 were purchased from Merck (Germany). All materials were used without further purification.

Preparation of SAP lyophilizate by solid-in-oil dispersion methods

The first step in this study was the preparation and optimization of O/W nanoemulsion base, by adding surfactant and co-surfactant components into the oil phase. VCO, Tween 80, and PEG 400 were the oil phase, surfactant, and co-surfactant, respectively, formulated in several concentrations and ratios. The addition of glycerin to the formula aimed to increase the solubility of SAP in the oil phase and was expected to affect SAP skin permeation. Each component was dispersed in deionized water and then stirred until homogenous dispersion was obtained.

The dispersion of SAP and amphiphilic molecules, i.e., lecithin (Lipoid S100) and PEG 20000, was achieved by mixing the SAP and amphiphilic molecules in ratios of 1:1 and 1:2, respectively. Each component was dispersed in deionized water and then stirred until a homogenous dispersion was obtained. Each mixture was frozen in liquid nitrogen and then lyophilized to form solid and semisolid lyophilizate of SAP-PEG 20000 and SAP-lecithin, respectively (Fig.1)[1,2,35]. SAP dispersion with lipophilic surfactant was initiated by forming water in an oil emulsion of SAP, VCO, and Span 80. The W/O emulsion was then frozen in liquid nitrogen and co-lyophilized until oily liquid lyophilizate was formed as SOD[1,2,3].

Characterization of Lyophilizate

The interactions between SAP and the amphiphilic molecules in the lyophilizates were characterized using FTIR spectroscopy (Agilent, Cary 630, USA). Samples was measures by

Attenuated total reflectance (ATR) technique which direct measuring reflected infrared beam when the beam comes into contact with a sample. Characterization included the indirect determination of SAP partitioned in the oil phase and morphology imaging using a Transmission Electron Microscope (TEM; JEOL JEM 1400, Japan). The amount of SAP in the aqueous layer was determined by HPLC (Knaeurs; PUMS 1000, UV VIS 2000, Germany) using a UV detector at 258 nm, Nucleosil amino column measuring 250 mm × 4 mm (Phenomenon) as the stationary phase and acetonitrile-phosphate buffer (45:55, 0.1 M, pH 2.5) as the mobile phase. The flow rate of the HPLC was 1 mL/min [7,15,16].

Preparation and Characterization of SAP Nanoemulsion

The nanoemulsion was prepared by adding SAP-PEG 20000, SAP-lecithin, and SAP-Span 80 lyophilizates into the VCO followed by the addition of the aqueous phase while continuously stirring. The resulting O/W nanoemulsion was homogenous and transparent. The nanoemulsion globule size and PDI were characterized by photon correlation spectroscopy (PCS) using a Delsa™ Nano Particle Analyzer (Beckman Coulter-USA). The samples were measured by dilution of nanoemulsion 10 times with deionized water and three times determination (triple). Physical stability of the nanoemulsion was observed at 25 °C and 40 °C and evaluated from the globule size of each nanoemulsion.

Skin Permeation Study of Nanoemulsion

In vitro skin permeation was evaluated using Franz diffusion cell employing snake skin (*Python reticulatus*) and Spangler's membrane. A sample (0.1 g) was attached to the membrane. Phosphate buffer (pH 7.4) was used as the receptor compartment solution. The temperature of the receptor compartment was maintained at 37±2 °C. The receptor compartment was continuously stirred to homogenize the solution. One milliliter of receptor solution was taken at 15, 30, 60, and 360 min and replaced with the same volume of phosphate buffer (pH 7.4). The concentration of SAP in the receptor solution was determined by HPLC. We evaluated the diffusion study of SAP solution and SAP nanoemulsion.

RESULTS AND DISCUSSION

It has been developed oil-in-water (O/W) nanoemulsion base containing virgin coconut oil (VCO), Tween 80, PEG 400, and phosphate buffer (pH 6) as the oil phase, surfactant, co-surfactant, and aqueous phase, respectively. The composition of the nanoemulsion base was optimized by varying ratios of surfactant and co-surfactant, i.e., 1:1, 2:1, and 3:1. The globule size of all nanoemulsions formula was less than 50 nm with a polydispersity index (PDI) of ≤ 0.5. These data corresponds to a good homogeneity of the dispersed globules. SAP showed the highest solubility in the oil phase with the addition of 10% (w/w) glycerin (Table 1).

Table 1. Solubility of SAP in The Oil Phase With theAddition of Glycerin

Component	Concentration (% w/w)		
	B1	B2	B3
SAP	1.5	1.5	1,5
Tween 80	17	17	17
PEG 400	9	9	9
Glycerin	0	5	10
VCO	3	3	3
Dissolved SAP*)	5.74 ± 0.32	38.08 ± 2.01	94.55 ± 0.26

*Presented as the mean of three measurements

These data is in accordance with the solubility of SAP in glycerin[6]. Thus, the nanoemulsion formula comprised of 10% (w/w) glycerin as well as the optimized surfactant and co-surfactant ratio. The small globule size of nanoemulsion is promising to deliver SAP for permeating the stratum corneum of the skin[9,17].

VCO was utilized in the oil phase because it consists of short-chain fatty acids such as lauric acid (C 12) and myristic acid (C 14) that improve the potential for VCO to readily mix with water in the O/W nanoemulsion system[18]. The optimization of the SAP nanoemulsion formula is shown in Table 2.

Table 2. Optimization of SAP Nanoemulsion Formula

Component	Concentration (% w/w)		
	C1	C2	C3
SAP	1	1	1
VCO	3	3	3
Tween 80	12	16	18
PEG 400	12	8	6
Glycerin	10	10	10
Distilled water	62	62	62
Globule size (nm)*	49.73±6.127	47.2±7.850	34.56±4.400
PDI*	0.340±0.004	0.344±0.004	0.370±0.002

*Presented as the mean of three measurements

The optimum ratio of SAP and amphiphilic molecules in SAP-PEG 20000 and SAP-lecithin was 1:1 (based on preliminary data). The mixing process of both dispersions comprised the interaction of physical mixture based on previous studies, while a different process was involved in the mixing process of SAP-Span 80, as a W/O emulsion formation. All of the mixtures were then followed by co-lyophilization[11,19]. The mixing process is described in Figure.2.

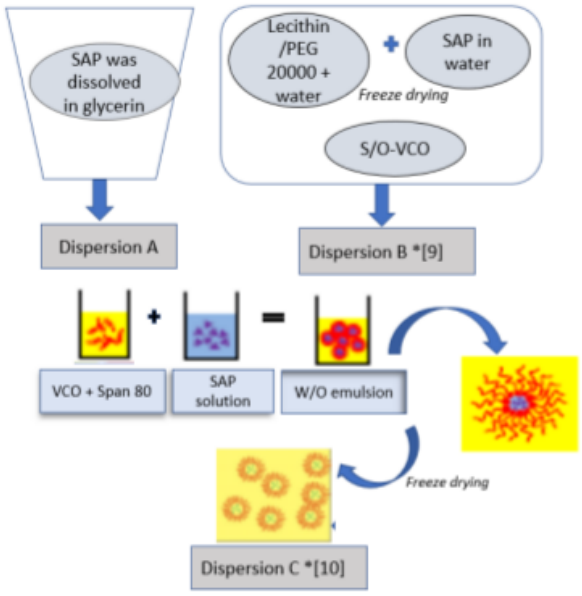


Fig. 2. Preparation procedure for S/O dispersion of SAP by three methods (Dispersion A: solution of SAP in glycerin, Dispersion B: liquid mixing of SAP in water and lecithin/PEG 20000 then co-lyophilization by freeze dry, and Dispersion C: emulsion of SAP-VCO-Span 80, then co-lyophilization by freeze drying process

The resulting lyophilizates were dry powder, sticky powder, and viscous oily liquid for L1 SAP-PEG 20000 (L1), SAP-lecithin (L2), and SAP-Span 80 (L3), respectively. Lyophilizates were stored in a desiccator to avoid hydration. The compositions of the lyophilizates are shown in Table 3.

Table 3. Composition of lyophilizates

Component	Concentration (% w/w)		
	L1	L2	L3
SAP	1	1	1
PEG 20000	1	-	-
Lecithin	-	1	-
VCO	-	-	3
Span 80	-	-	20

Characterization of the lyophilizates was undertaken by Fourier Transform Infra Red (FTIR) spectroscopy with the aim of identifying the interaction between SAP and amphiphilic polymers. The FTIR spectra are shown in Figure 3.

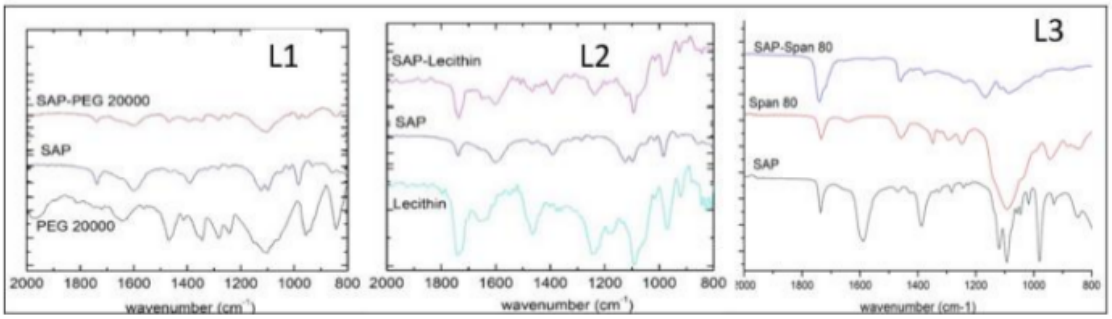


Fig. 3. FTIR spectra of lyophilizates L1, L2, and L3

The FTIR spectra confirm that molecular interaction did not occur between all of the lyophilizates, SAP with PEG 20000 as well as lecithin and Span 80. The data indicate that no new molecular compound was formed. The characteristic peaks of SAP in 1000-1100 cm^{-1} were observed in all mixtures. SAP formed a physical interaction with PEG 20000 while conjugation also occurred with ionic lecithin was suspected between phosphate group (positive) in SAP and cholin-amina group (negative) from lecithine. Based on the FTIR spectra, SAP was predicted to be more partitioned in the lecithin compared to PEG 20000 due to the more lipophilic nature of lecithin. Phosphate or ionic nitrogen compound may be ionized as a positive or negative ion, forming a barrier in an aqueous environment [16]. Thus, SAP was expected to be entrapped better in the lecithin compared to PEG 20000 and Span 80.

The partitioning of SAP in the lyophilizates was evaluated using an indirect method in the form of the determination of SAP in the aqueous phase by High-performance liquid chromatography (HPLC). The analytical method used was based on previous studies[7,15,16] and showed that SAP-lecithin (L2) exhibits a lower amount of SAP partitioning into the aqueous phase compared to the oil phase (43.79 ± 2.37) and is thus different to L1 (84.89 ± 12.37) and L3 (87.85 ± 4.25). Therefore, lecithin was better able to retain SAP in the oil phase than PEG 20000 or Span 80. The dispersion of SAP-lecithin in oil is expected to show optimum ability in terms of retaining SAP and this characteristic is essential for the penetration of SAP through the SC layer.

Lyophilizates were incorporated into VCO to form an O/W nanoemulsion using Tween 80, PEG 400, and phosphate buffer as the surfactant, co-surfactant, and aqueous phase, respectively (Table 4). The average globule size of SAP nanoemulsion is 50–200 nm. The globule size of the mixture was relatively higher than that in SAP nanoemulsion due to the influence of amphiphilic polymers and lipophilic surfactants that formed a barrier around SAP.

Table 4. Composition of SAP Nanoemulsion

Component	Concentration (% w/w)			
	F1	F2	F3	F4
SAP	1	-	-	-
L1	-	1	-	-
L2	-	-	1	-
L3	-	-	-	0.5
VCO	3	3	3	3
Tween 80	12	12	12	12
PEG 400	12	12	12	12
Glycerin	10	10	10	10
Phosphate buffer (pH 6)	63	63	63	63.05
Appearance	Clear liquid	Clear liquid	Clear liquid	Viscous liquid

An accelerated stability study was conducted by storing the nanoemulsion for 30 days at room temperature (25 °C) and in a climatic chamber (40 °C). Physical appearance and globule size were observed periodically. After 30 days at 25 °C, the average globule size of SAP-lecithin and SAP-PEG 20000 had increased to ± 200 nm. SAP-Span 80 showed a greater increase (±700-1200 nm) due to the high viscosity of Span 80 as a lipophilic surfactant. Storage in a climatic chamber at 40 °C increased the globule size to around ± 1000-1500 nm for SAP, SAP-PEG 20000, and SAP-lecithin, and to more than 2000 nm for SAP-Span 80 nanoemulsion. A two-way Anova statistical analysis confirmed that the globule size of each nanoemulsion was statistically different ($\alpha = 0.05$, $P < 0.05$) during the 30 days' storage at 25 °C and 40 °C. The globule size increased significantly at 40 °C due to the Ostwald ripening that commonly occurs in emulsion systems and is very often observed in an O/W emulsion system[8,20,21,22]. The increment of temperature caused flocculation of the globules and led to the size increment. Besides, globule or particle motility will be increased at a higher temperature as this promotes aggregation under hydrodynamic influence.

The nanoemulsion base shows spherical oil globules dispersed in the aqueous phase. A Transmission Electron Microscope (TEM) is able to capture the morphology of the nanoemulsion by emitting high voltage beams of electrons into the samples, which is then followed by focusing and magnifying the reflection through the lens. The resulting images are shown in Figure 4.

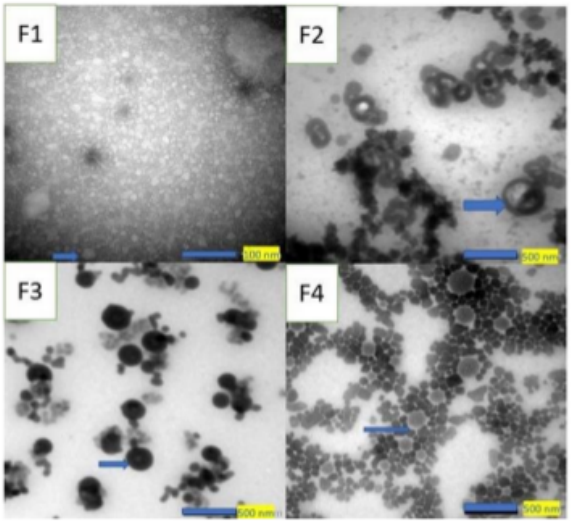


Fig.4. TEM imaging results of F1, F2, F3, and F4 nanoemulsion (Magnification 20000x)

The different morphologies show the lyophilizate nanoemulsions. The F3 (SAP-lecithin) and F4 (SAP-Span 80) nanoemulsions show more spherical features compared to the F2 (SAP-PEG 20000) nanoemulsion despite its inhomogenous globule size. SAP-lecithin nanoemulsion contains phospholipid partitioned in only a small amount into the aqueous phase and dominantly incorporated into Tween 80 and glycerin. Therefore, lecithin acts as the coating material for the active ingredient, forming a nanotope-like system[23]. However, we cannot be sure that the entrapped SAP in lecithin will not partition into the aqueous phase. SAP-PEG 20000 nanoemulsion consists of spherical and irregular non-spherical globules due to the hydrophilicity of PEG 20000 that has a high tendency to dissolve in the continuous phase of the nanoemulsion system, and there was steric hindrance of PEG 20000. SAP-Span 80 (F4) nanoemulsion was relatively denser than the other systems because the lipophilicity of Span 80 led to the formation of denser dispersed globules in the oil phase. The larger globule size occurred due to the aggregation of forming globules and the fact that the nanoemulsion is no longer physically stable.

A permeation study was conducted by an *in vitro* diffusion test using Franz diffusion cell with Spangler's membrane and snake skin (*Python reticulus*) as the skin membranes model. There is no significant difference in the thickness and lipid content of *shed snake skin* (SSS) and human skin. However, SSS has a lower water content than the SC layer of human skin[24]. The SAP solution, SAP nanoemulsion, and the mixture of SAP nanoemulsion and amphiphilic polymer and lipophilic surfactant were evaluated. A nanoemulsion system with small particle size is suitable for delivering hydrophilic molecules transcutaneously. For the permeation study, the samples were prepared by mixing about 1% of SAP lyophilizates into the nanoemulsion. As the reference, SAP nanoemulsions without lyophilization and SAP solution were prepared and evaluated. Testing for the amount of SAP that penetrated into the receptor compartment as determined by HPLC showed different results for each nanoemulsion. However, different membranes did not significantly affect the total amount of diffused SAP for each respective sample. The diffusion profiles are shown in Figure 5.

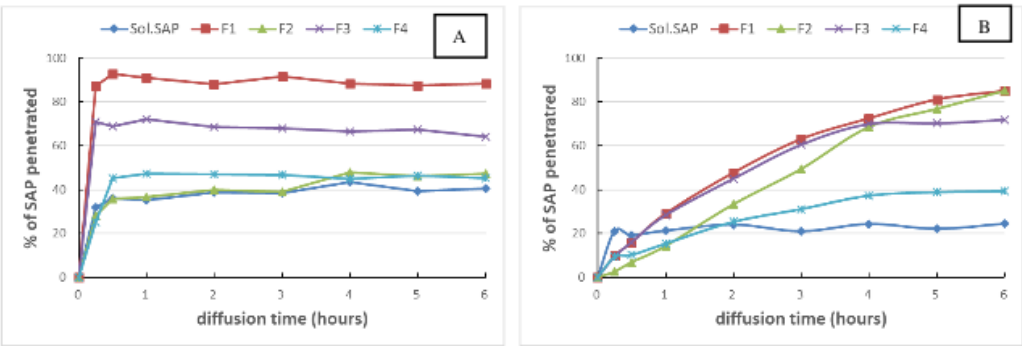


Fig. 5. Diffusion profiles of SAP nanoemulsions and SAP solutions as a control using SSS (A) and Spangler's membrane (B).

The amount of SAP penetrated across the SSS membrane at 1 hr was higher than the amount of SAP that crossed the Spangler's membrane due to the difference in the characteristics of the membranes. SSS membrane has a keratin-rich layer that has a similar structure to the keratinocytes of SC. Meanwhile, Spangler's membrane corresponds only to the intracellular lipid in the mortar of SC[25,26]. At 15 and 30 min., the diffusion across the SSS membrane of SAP in the nanoemulsion system and SAP-lecithin lyophilizate nanoemulsion showed higher profiles compared to other samples and this was followed by a steady-state phase up to the 6-hour mark. Different profiles were exhibited for the Spangler's system, in which constant increments of SAP were observed for all formula starting from 15 min. and lasting through to the 6-hour point. Diffusion results showed that SAP nanoemulsion (F1), followed by SAP-lecithin (F3), SAP-PEG 20000 (F2), and SAP-Span 80 (F4) could penetrate and there were variation influenced by process and formula. SAP nanoemulsion without lyophilization showed the highest penetration because the SAP was expected to exist as a free form and the addition of glycerin increased the amount of SAP to cross the SC layer. But the reason using a SOD system and combining SAP-lecithin system tended to have higher penetration than SAP-PEG 20000 and SAP-Span 80 because the lipophilicity of lecithin improves its ability to cover SAP and enhances the penetration ability of SAP through the SC barrier. This results also proven that when active substance or drug molecules penetrate through SC layers, it can pass through the deeper epidermis and enter the dermis tissue[27,28]. A high-molecular-weight polymer such as PEG that is hydrophilic tends to make SAP dissolve in the aqueous phase and Tween 80 as surfactant on nanoemulsion formulas which is have a long chain hydrocarbon could acts as a triggers to improve the release of active molecules into the skin layers[28]. The SAP-Span 80 nanoemulsion system has the lowest penetration profile, probably due to the partitioning of SAP into the aqueous phase during the mixing of the lyophilizates with the nanoemulsion.

SAP in the phosphate buffer (SAP solution) has lower diffused SAP than SAP in the nanoemulsion. The low value of SAP log P (-0.005) indicates the hydrophilicity of SAP and its difficulty in crossing the SC layer. A two-way Anova confirmed that each SAP formula has statistically different diffusion profiles in both membranes ($\alpha = 0.05$, $P < 0.05$).

Based on these phenomena, nanoemulsion formation with amphiphilic molecules and lipophilic surfactants, as well as the addition of glycerin, play an important role in improving the

transcutaneous delivery of hydrophilic compounds. This method is promising in relation to enhancing the skin permeation of small molecules.

CONCLUSION:

Sodium ascorbyl phosphate, a hydrophilic compound, offers the potential for transcutaneous delivery through a nanoemulsion system and the addition of glycerin and dispersion with amphiphilic molecules and lipophilic surfactants. The SAP-lecithin mixture system showed greater permeation compared to SAP-PEG 20000 and SAP-Span 80.

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