

Formulation of ophthalmic vitamin A nanoemulsion with Kolliphor® EL as surfactant and transcutool as cosurfactant and hen's egg test chorioallantoic membrane (HET CAM) irritation test

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Abstract Conventional eye drop preparations are the main choice for the treatment of eye disorders; however, they have low penetration and bioavailability. Vitamin A is nonphotostable, lipophilic, and easily degraded in the presence of oxygen. This study aimed to characterize a vitamin A ophthalmic nanoemulsion without any irritation. The nanoemulsion was prepared using a mixture of surfactant Kolliphor® EL and cosurfactant Transcutol (S_{mix}) for F1 (1:1), F2 (1:2), and F3 (2:1). The formulas were examined by pH, optical transmission, refractive index, particle size, PDI, and Hen's Egg Test Chorioallantoic Membrane (HET-CAM) irritation tests. The selected formula was F3, with the best optical transmission ($97.35 \pm 0.173\%$), pH (4.94 ± 0.04), refractive index (1.3534 ± 0.0002), particle size (24.47 ± 1.888 nm), PDI (0.353 ± 0.02), and no irritation response. This formula shows a good potential for ophthalmic eye drop preparation.

1 Introduction

Vitamin A deficiency remains a global health concern. In the long term, it can cause bacterial resistance, anemia, and xerophthalmia (dry eye), leading to [1]. In Riau, Indonesia, the incidence of xerophthalmia is 27.5%, and continues to increase [2]. Conventional eye drops are the most common therapeutic dosage forms in the market for eye disorders. However, low drug penetration and short retention time due to rapid tear turnover and difficulty of drug diffusion through the multi-layered structure of the eye, result in a low bioavailability that less than 5% [3,4]. Vitamin A is lipophilic and easily degraded by oxygen. To improve its solubility and physical stability, vitamin A was formulated as a nanoemulsion. A nanoemulsion is a dispersion system consisting of an oil phase, a water phase, and a surfactant that forms stable nano-globules [5]. The nanoemulsion form can improve the

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bioavailability of eye drop preparations because the nano size can increase the permeation of the drug into the cornea, provide sustained drug release, and reduce systemic side effects [6]. The proper selection of surfactant and cosurfactant components can affect the globule size of the resulting nanoemulsion. The HLB value is an important criterion for surfactant selection. The HLB value required for nanoemulsions with M/A systems is greater than 10 [7]. Kolliphor® EL with 13.5 HLB value has often been investigated owing to its better emulsifying capacity than the Tween series. In addition, Kolliphor's branched alkyl chain structure with lower ethoxylation level makes it penetrate oil phase effectively [8,9]. Transcutol is a nonionic surfactant that can sterically stabilize nanoemulsions by forming a coat around their surface. Non-ionic surfactants do not contribute charge to the nanoemulsion globule; therefore, they do not affect the stability of the nanoemulsion formed. The purpose of this study was to determine the ophthalmic preparation formula of vitamin A nanoemulsion with a combination of surfactant Kolliphor® EL and co-surfactant transcutol based on the characterization results, which were then tested for safety through the Hen's Egg Test Chorioallantoic Membrane (HET-CAM) irritation test [10]. This research is in accordance to one of the agendas of sustainable development goals (SDGs), namely to ensure healthy lives and promote well-being for all.

2 Material and Methods

2.1 Materials

Vitamin A palmitate raw material (USP units/g 1.100.000, Sigma Aldrich, Germany), Kolliphor® EL (Sigma Aldrich, Germany), Transcutol (provided by PT Rohto Laboratories Indonesia), Ethyl oleate (Merck, Germany), Cethalconium chloride/CKC (Sigma Aldrich, Germany), Sodium metabisulfite (Merck, Germany), and Aqua pi (PT. Widatra Bhakti, Indonesia), alcohol 70% technical grade, NaOH 0,5 M, propylene glycol dan acetone), Sterile NaCl 0,9% pi (PT. Widatra Bhakti, Indonesia), and Navitae eye drop.

2.2 Methods

2.2.1 Preparation of vitamin A nanoemulsion

The formula for the Vitamin A nanoemulsions is shown in Table 1. Vitamin A, Smix and ethyl oleate were vortexed for 30 seconds in an amber bottle (oil phase). Cetalkonium chloride and sodium metabisulfate were dissolved in Aqua pi (water phase). The water phase was slowly dripped and stirred using a magnetic stirrer (Thermo Fisher Scientific, USA) at room temperature (25 ± 2 °C) with a stirring speed of 6 for 2 h. The nanoemulsion was characterized according to these parameters, and the best formula was selected based on the specifications listed.

Table 1. The composition of Vitamin A nanoemulsion

Materials	F1 (% w/v)	F2 (% w/v)	F3 (% w/v)
Vitamin A	0.1	0.1	0.1
Etil Oleat	2	2	2
S _{mix} (Kolliphor® EL : Transcutol = 1:1)	15	0	0
S _{mix} (Kolliphor® EL : Transcutol = 1:2)	0	15	0
S _{mix} (Kolliphor® EL : Transcutol = 2:1)	0	0	15
Cethalconium chloride	0.2	0.2	0.2
Sodium metabisulfite	0.1	0.1	0.1
Aqua pi	ad 100	ad 100	ad 100

Table 2. Nanoemulsion parameter test and specification

Parameter	Specification
Optical Transmission	> 90%, transparent [11,12]
Refractive index	<1.476 [13]
pH	3.5–8.5 [14]
Z-average and PDI	10-200 nm, <0.5 [15]
HET-CAMP	No irritation response [16]

2.2.2 pH, refractive index, and optical transmission characterization

The pH meter (OHAUS® Starter 300) was calibrated using standard buffers of pH 4.0, 7.0, and 10.0, before used for sample measurement [17]. Refractive index measurements were performed using an ABBE Refractometer (ATAGO® DR-A1-Plus). The refractometer was calibrated first using distilled water, then 1-2 drops of sample were dripped on the optical lens, and the refractive index reading was taken. Quartz cuvettes were used to measure the optical transmission. The sample (300 µL) was diluted at a ratio of 1:10 using aqua pi at a wavelength of 520 nm with a UV-Vis spectrophotometer (UV-1800 Shimadzu Corporation). The sample was declared transparent if %T>90%, translucent if 10%>T%<90% and turbid if %T<90% [11,12]. Analytical statistics of characterization data were analyzed using GraphPad Prism software version 10.0.1 (170) with the Shapiro-Wilk normality test. If the results of the Shapiro-Wilk normality test were normally distributed ($p > 0.05$), the parametric one-way analysis of variance (ANOVA) test was continued. However, if the results showed that the data were not normally distributed ($p < 0.05$), the Kruskal-Wallis non-parametric test and Wilcoxon (Mann–Whitney) test were performed. Data are presented as the mean of three independent experiments ($n = 3$), standard deviation (SD), and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ were considered significant when compared with each group. Statistical significance was set at $P > 0.05$.

2.2.3 Particle size and polydispersity index measurement

Particle size and polydispersity index (PDI) measurements were limited by the best formula. The globule size and PDI were determined using a Malvern® Zetasizer NS at 25 ± 1 °C, used a glass cuvette with a square aperture (PCS1115). A 100 µL of Vitamin A nanoemulsion formula was diluted 10-fold with aqua pi before measurement [15].

2.2.4 Hen's egg test-Chorioallantoic membrane (HET–CAM) Irritation test

The irritation response was measured using Hen's egg test-chorioallantoic membrane (HET-CAM) method. Freshly fertilized leghorn chicken eggs were incubated at 37 ± 0.5 °C for 5 days with RH ($40 \pm 4\%$). During incubation, the eggs were kept horizontal and rotated daily to provide a good position for the embryo and prevent it from sticking to one side of the egg. On the 5th day, the air cavity in the egg was marked to clean the part of the eggshell to be opened using 70% alcohol. Then open the shell using scissors and tweezers that sterilized using 70% alcohol. After the eggshell was opened, the outer membrane of the egg was moistened using sterile 0.9% NaCl solution, incubated for 10 min, and the eggs that did not have CAM damage were selected. Drip 0.2-0.3 mL of the control and sample solutions onto the CAM surface and left for 20 s. The CAM surface was observed for the irritant effect of the sample and the control for 5 min. CAMs were scored according to Table 3 and the interpretation of Table 4 [16].

Table 3. Time-dependent numerical scores for each of the three irritant responses

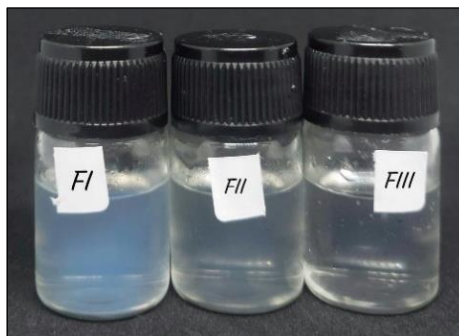
Category	Score		
Effect/Time (min)	0,5	2	5
Hyperemia	5	3	1
Hemorrhage	7	5	3
Clotting/coagulation	9	7	5

Table 4. Irritation potential interpretation of various cumulative scores

Cumulative score	Irritation assessment
0-0.9	None
1.0-4.9	Slight
5.0-8.9	Moderate
9.0-21.0	Severe

3 Results and Discussion

The method used in the preparation of Vitamin A nanoemulsion uses the low-energy titration method. The low-energy titration method produces small droplets through changes in the composition or temperature that pass through a low surface tension point. This process involves the Phase Inversion Temperature (PIT) or Phase Inversion Composition (PIC), which occurs when the A/M system changes to M/A. In this study, nanoemulsions were prepared by slow titration of the aqueous phase (CKC, sodium metabisulfate, and aqua pi) into the oil phase (vitamin A, S_{mix} , and ethyl oleate) at room temperature ($25 \pm 2^\circ\text{C}$), which caused a change from A/M to M/A during aqueous phase titration [18]. The vitamin A nanoemulsion was prepared using three formulas with variations in the ratio of Kolliphor® EL (surfactant) and Transcutol (cosurfactant), namely F1 1:1, F2 1:2, and F3 2:1. The variation in the amount was used to determine the S_{mix} component that produced the selected formula with the best nanoemulsion characteristics. The results of nanoemulsion preparation using the three formulas are shown in Fig. 1. The figure shows that the clearest formula obtained is shown in F3, followed by F2 and F1, which look less clear/translucent. Clear preparations are related to the constituent components, small globule droplet size, and the Refractive Index. The preparation can appear clear or transparent if it has a globule diameter < 50 nm or translucent with a globule size range of $50 \text{ nm} < \text{diameter} < 200 \text{ nm}$ [17].

**Fig. 1.** The visual of ophthalmic vitamin A Nanoemulsion preparation of F1, F2, and F3

3.1 Optical Transmission

Optical Transmission describes the clarity of the preparation used to estimate the occurrence of visual disturbances when applied to the eye [12]. Clarity is also a parameter for the perfect dispersion of the nanoemulsion system [19]. The optical transmission test results are shown in Fig. 2. Percent transmittance value that is close to 100% indicates a clear dispersion, and it is estimated that the size of the oil droplets reaches nanometers. Based on the Fig. 2, the highest percent transmittance was in F3 (97.35%), followed by F2 (95.53%), and F1 (84.95%), so that those that meet the target specifications are F2 and F3 [19]. In addition, the results of the analytical statistics performed that changes in the ratio of surfactants and cosurfactants can cause significant differences in the percent transmittance in each formula. Based on the required HLB of ethyl oleate, which is 10.8 ± 0.8 , a mixture of surfactants and cosurfactants that is close to this value is needed [20]. The HLB value of Kolliphor® EL is 13.5 and transcutool is 4.2 [16,21]. Based on the calculations in equation 1 and 2, to obtain an HLB value that is close to the required HLB of ethyl oleate, 67.35% Kolliphor® EL and 32.65% Transcutol are needed. This amount is close to the ratio of Kolliphor® EL to Transcutol in F3, which is 2 :1. The combination of surfactants and cosurfactants in the preparation of nanoemulsions is essential to produce the very low interfacial tension required to reduce the droplet size to the nanometer range. Surfactants adsorb at the oil/water interface, causing a decrease in surface tension and leading to a reduction in droplet size and the formation of NE [16]. If the size of the globules in the emulsion is less than 100 nm, light can penetrate without scattering, resulting in a clear visual preparation [22].

$$Kolliphor® EL (\%) = \frac{(HLB \text{ required ethyl oleat} - HLB \text{ Transcutol})}{(HLB \text{ Kolliphor® EL} - HLB \text{ Transcutol})} \times 100\% \quad (1)$$

$$= \frac{(10.8 - 4.2)}{(14 - 4.2)} \times 100\% = 67.35\%$$

$$Transcutol(\%) = 100\% - \% Kolliphor® EL \quad (2)$$

$$= 100\% - 67.35\% = 32.65\%$$

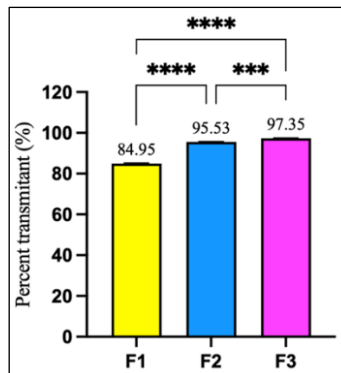


Fig. 2. Percent transmittant result of vitamin A nanoemulsion

3.2 pH measurement

pH testing was carried out to ensure that vitamin A nanoemulsion preparations were in accordance with the physiological conditions in the eye [13]. If the pH of the preparation is too far from the physiological conditions of the eye, irritation can occur. The preparation is performed in the pH range of 3.5-8.5, because tears can buffer the preparation so that can be

tolerated by the eye [14]. As shown in Figure 3, the pH values were F1 5.34, F2 5.22 and F3 4.94. Morsi et al. (2017) showed that the pH of the nanoemulsion acetazolamide eye drop preparation formula was in the range of 4.9-5.5 and showed no irritation when applied to the eye [12]. Kolliphor® EL has a pH of 6.0-8.0 [23]. Transcutol has a pH of 4.0-7.0 [24]. Changes in the surfactant and cosurfactant components can alter the pH of each formula. Based on the statistical analysis test results, differences in the composition of S_{mix} caused significant changes in the pH. Nevertheless, all the formulas still meet the expected target range.

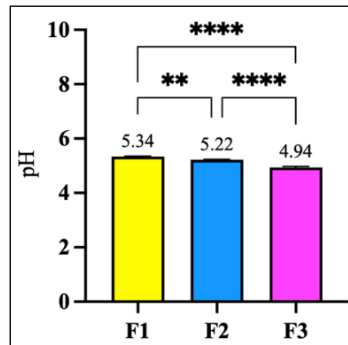


Fig. 3. pH result of vitamin A nanoemulsion for F1, F2, and F3

3.3 Refractive index

Figure 4 shows the average refractive index of F1 (1.355), F2 (1.354), and F3 (1.353). The three vitamin A nanoemulsion formulas meet the specification for ophthalmic preparations from 1.340-1.360 that in line with the physiological of tears, which is < 1.476 [13]. A high Refractive Index in eye preparations can cause visual discomfort owing to distortion or changes in vision when eye drop preparations are used [11]. In addition, Figure 4 shows that the change in the S_{mix} composition impacted a significant difference in the refractive index, except in F2 against F3.

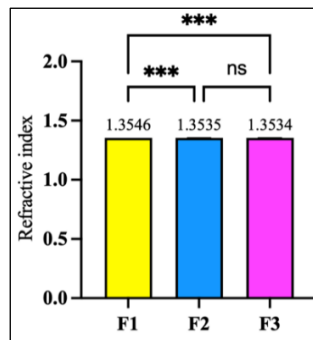


Fig. 4. Refractive index of vitamin A nanoemulsion for F1, F2, and F3

3.4 Particle size dan polydispersity index (PDI)

F3 is the selected formula because it meets the requirements of nanoemulsion preparation with the highest percent transmittance and the lowest refractive index. Particle size and polydispersity index (PDI) testing was only carried out on the selected formula to further verify that the selected vitamin A nanoemulsion formula meets globule size nanometer scale

[25]. In addition, a small globule size is very important for ophthalmic preparations because it can increase drug permeation and distribution to eye's tissues, thereby increasing bioavailability and therapeutic effects [17].

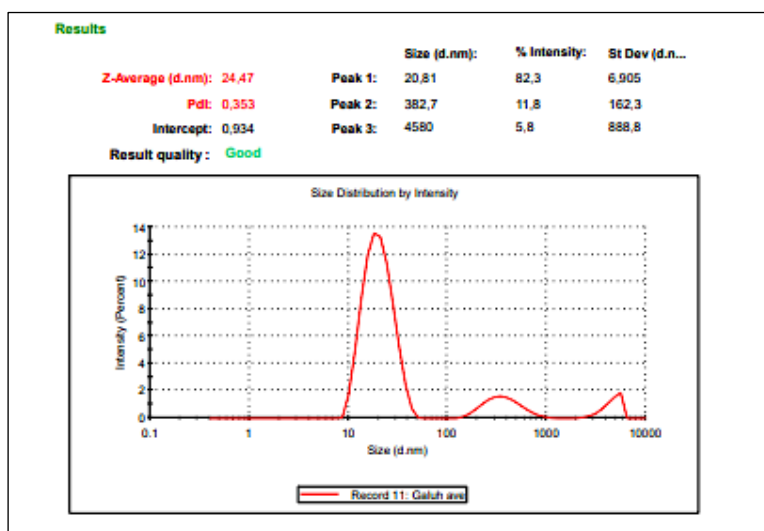


Fig. 5. Particle size dan polydispersity index (PDI) result of F3

Figure 5 shows one of the measurement results for particle size and polydispersity index (PDI). The particle size was 24.47 ± 1.888 nm and PDI was 0.353 ± 0.02 . These results are in accordance with the specifications which can form a nanosystem with a globule size of 10-200 nm [26]. This is in line with research conducted by Morsi et al. (2017) that nanoemulsion preparations with a %T value $\geq 90\%$ are estimated to have a globule size between 10-200 nm [12]. The globule size of the nanoemulsion can be attributed to the use of the right mixture of surfactants and co-surfactants. The higher the surfactant concentration, the smaller are the globules produced. This is because surfactants can lower the surface tension of the emulsion, allowing energy to break down and shrink globules. Cosurfactants play an important role in assisting surfactants by filling the gaps between surfactant molecules, resulting in smaller and more stable globules [27,28]. The dispersity of the nanosystem can be determined from the PDI value. The PDI value of F3 satisfied the target value of less than 0.5. The PDI value should ideally be below 0.5 to show the homogeneity of the globule size with the nano system. Homogeneity means that the produced nanoemulsion globules are uniform, so they are more stable and not easily aggregated physically. High nanoemulsion stability and homogeneity can increase the bioavailability [28].

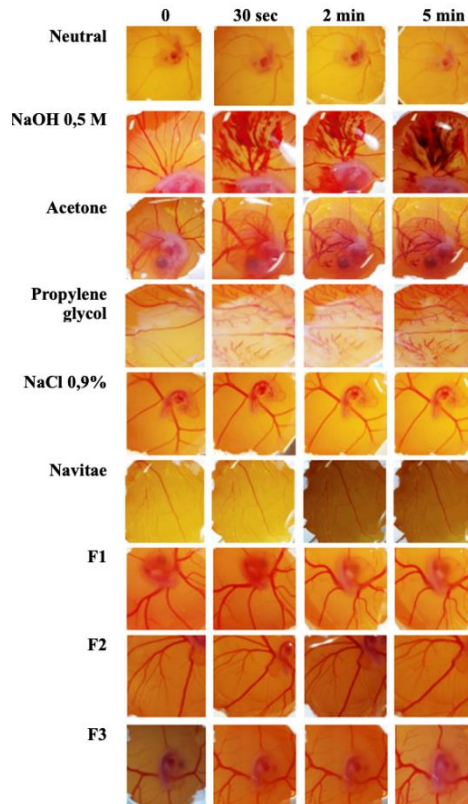
3.5 Hen's Egg Test Chorioallantoic Membrane (HET-CAM) irritation test result

The irritation test is a toxicity test that can be used to estimate the irritative potential of a material that can be applied to the eye. The Hen's Egg Test Chorioallantoic Membrane (HET-CAM) method is an alternative to the Draize method on rabbit eyes. The HET-CAM method uses egg CAM vascular tissue that can respond to inflammatory effects, as in rabbit eyes [14]. Tests were carried out on CAM without treatment, positive controls consisting of 0.5 M NaOH (coagulation), propylene glycol (hyperemia) and acetone (hemorrhage), negative control (NaCl 0.9%), conventional vitamin A products on the market (Navitae eye drop), as well as F1, F2 and F3. The results of the HET-CAM test can be seen in Figure 6 and the scoring results can be seen in Table 5.

Table 5. Cumulative score of HET-CAM test

Sample	Cumulative score (Average \pm SD)	Irritation assessment
NaOH	19 ± 2	Severe
Propilenglikol	4.33 ± 1.15	Slight
Acetone	14.33 ± 1.15	Severe
Navitae	0 ± 0	None
NaCl 0,9%	0 ± 0	None
F1	0 ± 0	None
F2	0 ± 0	None
F3	0 ± 0	None

The raw materials of ophthalmic nanoemulsion vitamin A preparations consisting of ethyl oleate, Kolliphor® EL, and transcutol are commonly used for ocular nanoemulsion formulation. Therefore, that was expected to be safe for formulated eye drops [17]. Figure 6 and Table 5 show that all positive controls showed a positive response of Severe category coagulation in NaOH 0.5 M with a score of 19 ± 2 , hyperemia in propylene glycol with Slight category with a score of 4.33 ± 1.15 and Severe category hemorrhage in acetone. NaCl 0.9% as a negative control showed no irritation response until the end of the observation period, including Navitae, F1, F2, and F3. Based on these results, the change in S_{mix} composition in the three formulas did not affect the safety of the preparation.

**Fig. 6.** HET-CAM test of neutral condition, positive control, negative control, F1, F2, and F3

4 Conclusion

Formula 3 with the ratio of surfactant (Kolliphor® EL) and cosurfactant (Transcutol) 2:1 was the selected formula with the characterization results of Optical Transmission (97.35 ± 0.173), pH (4.94 ± 0.04), Refractive Index (1.3534 ± 0.0002), particle size (24.47 ± 1.89) and PDI (0.353 ± 0.02). In addition, Formula 3 did not cause an irritation response based on the results of the irritation test with the Hen's Egg Test Chorioallantoic Membrane (HET- CAM) method. The selected vitamin A nanoemulsion formulas have the potential to be further developed into safe, bioavailable, and high-quality ophthalmic preparations.

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