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Original Article

DEVELOPMENT OF TRANSDERMAL NANOEMULSION FORMULATION FOR SIMULTANEOUS DELIVERY OF PROTEIN VACCINE AND ARTIN-M ADJUVANT

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ABSTRACT

Objective: Vaccine used to enhance immune response is commonly administered via parenteral route. However, it shows some disadvantages. This research aimed to develop a non-invasive vehicle for administering an immunogenic protein and a lectin adjuvant via transdermal route. The hydrophilic nature of protein and lectin became the major challenge faced in this study.

Methods: Bovine serum albumin (BSA) was used as protein model and artin-M isolated from cempedak (*Artocarpus integrifolia*) seed as adjuvant model. Nanoemulsion was formulated as oil in water type and prepared by self-nanoemulsification method. The formulation consisted of virgin coconut oil (VCO), Tween 80, and PEG 400 as oil phase, surfactant, and co-surfactant, respectively. BSA and artin-M were incorporated into the oil phase as co-lyophilized solid dispersion in PEG 20000. The nanoemulsion was characterized by BSA entrapment efficiency, hemagglutination activity of artin-M, and stability testing using freeze thaw conducted by alternating the storage at room temperature and 4 °C up to 6 cycles.

Results: The addition of PEG 20000 in solid dispersion at the rasio of 1:1 to BSA markedly increased BSA entrapment efficiency in oil phase to 98.6 ± 0.15%. Artin-M incorporated using the same method was also maintained its activity as shown by hemagglutination testing. The freeze thaw testing revealed the stability of nanoemulsion stored in the fridge with no-aggregation observed. The globule diameter was maintained at the size of 42.7 ± 0.9 nm with polydispersity index of 0.321 ± 0.01.

Conclusion: The nanoemulsion is potensial as simultaneous transdermal delivery of protein vaccine and lectin adjuvant.

Keywords: Artin-M, BSA, Nanoemulsion, Transdermal, Vaccine.

INTRODUCTION

A vaccine is any preparation intended to produce immunity to a disease by stimulating the production of antibodies. Vaccine is commonly administered via parenteral route, however, it shows some disadvantages, such as patients inconvenience, pain and swelling at injection site, and necessity of isotonicity and sterility [1,2].

Nowadays, it has been developed various methods of administering vaccines, one of which is through topical preparations. Skin is one of potential target for vaccine delivery due to existence of dendritic and Langerhans cells which are responsible in stimulating immune system for antigen elimination [3,4,5]. Vaccines can be administered via transdermal route. This route posseses more advantages than parenteral route, such as painless, no requirement of isotonic and sterile preparation, and no dependency to medicians upon administration [3]. However, there is a limitation for high molecular weight molecule such as protein to penetrate through stratum corneum. It becomes the main challenge in transdermal immunization. Currently, many researches have been developed transdermal vaccines delivery enhancement in the form of nano-size particulate, such as liposomes, nanoemulsions, and nanoparticles [1]. Nanoemulsion is one of interesting vehicles, which is mostly developed to enhance *in vitro* and *in vivo* absorption and bioavailability of drug through skin [6,7,8,9].

Another issue faced in protein vaccination is low immunogenic stimulation. One of alternative adjuvants having immunostimulatory effect is Artin-M, which is a D-mannosa-binding lectin isolated from jack fruit (*Artocarpus integrifolia*) seeds [10,11]. Based on the latest research, artin-M has been shown as a potent adjuvant that markedly increased the production of specific total IgG and IgG2a/ IgG1 rasio against *Neurospora canicum* at 1 µg/dosage of administration [12]. Another study showed the activity of artin-M, also known arthocarpin, to induce the synthesis of IgG1 in rat irrespective of the dose [13]. Artin-M was selected as adjuvant model because the hydrophilic nature of both protein and lectin would require similar strategy for skin penetration. In this research, both protein and lectin were incorporated into the oil phase of nanoemulsioan as nanosize globules to improve skin penetration and reach skin APC population. Therefore, this study was conducted to develop transdermal nanoemulsion formulation appropriate for simulataneous delivery of bovine serum albumin (BSA) as antigenic protein vaccine model and artin-M as adjuvant.

MATERIALS AND METHODS

Materials

Virgin coconut oil (VCO, purchased from SITH Bandung), Tween 80, PEG 400, and trichloro acetic acid (TCA) (Merck, Germany), bovine serum albumin (BSA), phosphate buffer saline (PBS), Bradford reagent (Sigma Aldrich, Singapore), artin-M (SF ITB laboratory), PEG 20000 (Fluka, Singapore), human erythrocytes (O group) (Red Cross, Indonesia)

Preparation of oil in water spontaneous nanoemulsion base formulation

A O/W nanoemulsion was formulated using virgin coconut oil (VCO), Tween 80, and PEG 400 as oil phase, surfactant, and co-surfactant, respectively. The formulation was optimized in two steps. Firstly, optimization of total amount of surfactant and cosurfactant that was carried out by combining Tween 80 and PEG 400 at a fixed ratio of 1:1 with the total amount was varied as shown in Table 1.

Secondly, optimization of the rasio of Tween 80 and PEG 400 which was varied as shown in Table 2. The mixture of VCO, Tween 80 and PEG 400 was added deionized water to provide 3% w/w of VCO in final nanoemulsion. The mixture was then stirred gently (200 rpm) until a clear nanoemulsion was produced. The optimum formulation was selected as the clear nanoemulsion having the smallest globule size and the most stable upon centrifugation testing at the speed of 3750 rpm for 5 hours.

Table 1: Optimization of nanoemulsion formulation containing various total amounts of surfactant and cosurfactant

Materials	Amount of material (% w/w)				
	A1	A2	A3	A4	A5
VCO	3	3	3	3	3
Tween 80	10	12	14	15	19
PEG 400	10	12	14	15	19
Water	77	73	69	67	59

Table 2: Various ratio of surfactant and cosurfactant at the total concentration of 24% w/w of nanoemulsion

Materials	Amount of material (% w/w)			
	B1	B2	B3	B4
VCO	3	3	3	3
Tween 80	8	6	18	16
PEG 400	16	18	6	8
Water	73	73	73	73

Preparation of BSA and Artin-M spontaneous nanoemulsion

In order to improve the dispersibility and avoid the aggregation of both BSA and artin-M in the oil globules of nanoemulsion, they were formed as solid dispersion with addition of an amphiphilic polymer (PEG 20000) prior to their incorporation into the oil phase. To maintain protein and lectin stabilities during the storage of nanoemulsion, it was proposed to prepare non-aqueous nanoemulsion concentrate that was reconstituted in water for injection (WFI) upon administration. Therefore, BSA and artin-M containing nanoemulsion was prepared by initial co-lyophilization process of both substances and subsequent incorporation into nanoemulsion base.

Co-lyophilization of BSA and Artin-M using PEG 20000

Solid dispersion of BSA and artin-M in PEG 20000 was prepared by co-lyophilization using procedur modified from the previous report [14]. Co-lyophilization of BSA and artin-M with PEG 20000 was carried out by dissolving PEG 20000 and BSA in deionized water at the mass ratio of BSA to PEG 20000 of 1:1, 1:2, 1:3, and 1:4 and artin-M at the mass ratio of Artin-M to PEG 20000 of 1:1. The aqueous admixtures were then frozen at -70 °C and lyophilized using a freeze-dryer (Teltstar LyoQuest) for 24 hours. The lyophilizate powder as solid dispersion of BSA-PEG 20000 and BSA-artin-M-PEG 20000 were kept in the fridge until used.

Incorporation of BSA and artin-M into nanoemulsion

In brief, BSA-artin-M-PEG 20000 solid dispersion was dissolved in a mixture of Tween 80 and PEG 400 in the oil phase by sonification (VirSonic 300, A=30%) for 6 cycles with 5 minutes/cycle. The spontaneous nanoemulsion was prepared as mention previously by adding deionized water with gentle stirring.

BSA Entrapment Efficiency Test

BSA entrapment efficiency test was determined indirectly by measuring unentrapped BSA in oil phase. Firstly, BSA was dispersed in oil phase, and then emulsified using 2% of TCA to form nanoemulsion system. Nanoemulsion was then centrifugated and the precipitated BSA was washed three times using 2% TCA. The precipitated BSA was dissolved in 1 mL of water. Of 800 µL BSA solution was reacted with 200 µL Bradford reagent, incubated for 5 minutes and the absorbance was measured using spectrophotometer at the wavelength of 595 nm. Standard calibration curve was constructed using BSA standard to calculate the amount of unentrapped BSA. BSA entrapment efficiency was determined using equation:

$$\frac{\text{The amount of BSA added} - \text{the amount of undentrapped BSA}}{\text{The amount of BSA added}} \times 100\%$$

Artin-M Activity Measurement by Hemagglutination Test

This test was aimed to determine the completion of artin-M entrapment in oil phase and evaluate the stability of artin-M in nanoemulsion. It was conducted by comparing the activity of artin-M before and after incorporation into nanoemulsion. Nanoemulsion was diluted twice using PBS. Subsequently, 2% of erythrocytes aqueous dispersion was added and then incubated for 2 hours at room temperature. The artin-M activity in nanoemulsion was determined by observing the minimal amount of lectin resulting in the last visible erythro-agglutination of human erythrocytes (O group). The activity of artin-M solution was used as the positive control. The test was also carried out for blank nanoemulsion and BSA nanoemulsion.

Physical Stability Test of Nanoemulsion

Physical stability test of nanoemulsion containing BSA and artin-M was analyzed by accelerated test using *freeze and thaw* test. Non-aqueous nanoemulsion concentrate containing BSA and artin-M was kept in glass vials and stored at temperature of 4 ^{o}C for 48 hours followed by storage at room temperature for 48 hours to complete one cycle. It was repeated until 6 cycles with intermittent storage at 4 ^{o}C and room temperature for the total 24 days. At the end of each cycle, the sample was reconstituted with deionized water and the nanoemulsion stability was analyzed by measuring globule size, polydispersity index, viscosity, zeta potential, and pH. Another accelerated testing was also performed on reconstituted nanoemulsion by centrifugation at 3750 rpm for 5 hours with sample withdrawl done every 1 hour interval [14]. The globule size and polydispersity index of nanoemulsion were determined by photon correlation spectroscopy (PCS) using Delsa™ Nano C Particle Analyzer (Beckman Coulter) at a fixed angle of 90 °C at temperature of 25 °C. Zeta potential was determined by electrophoretic light scattering using the same equipment.

Statistical analysis

Statistical significance of all data was assessed using one-way analysis of variance (ANOVA) with Tukey multiple comparison test. All the data were in triplicate and P-values <0.05 were considered as significant.

RESULTS AND DISCUSSION

Optimization of nanoemulsion formulation

The nanoemulsion was targeted to possess globule size around 40-300 nm, which is considered safe and can permeate stratum corneum for drug delivery through skin [16]. Nanoemulsion base consisted of VCO, Tween 80, PEG 400, and water. VCO was used as oil phase because it contains short-chain fatty acid, such as lauric acid and myristic acid which are consisted of 12 and 14 carbon chain, respectively. Because of this compostion of short-chain fatty acid, VCO is slightly hydrophile [17] and makes it suitable to be used as oil phase in O/W nanoemulsion. Tween 80 was used as emulsifying agent due to it's suitability for O/W emulsion and possesses low toxicity on human. PEG 400 was used as cosurfactant to stabilize the film on nanoemulsion globules.

The best formula of nanoemulsion was selected from the lowest content of surfactant and cosurfactant that can produce the smallest globule size. It was done through 2 steps of optimization, firstly at various total amont of surfactant and cosurfactant and secondly at various rasio of these components. Table 3 shows the result of the first optimization. The clear nanoemulsion with the smallest globule size was resulted from the total concentration of surfactant and cosurfactant of 24% w/w at the minimum. However, the globule size of nanoemulsion was getting increased at the total concentration of 30% w/w and 38% w/w. This result implied the formation of tubular micelles that was recognized by Delsa™ Nano C Particle Analyzer as larger globule size.

Table 3: Nanoemulsion characterization at various total amount of surfactant and cosurfactant

Formula	Total amount of surfactant (%)	Visual appearance	Globule size (nm)	Stability after centrifugation
A1	20	Cloudy	-	-
A2	24	Clear	29.1 ± 0.93	Stable
A3	28	Clear	29.2 ± 0.54	Stable
A4	30	Clear	32.9 ± 1.1	Stable
A5	38	Clear	48.5 ± 1.35	Stable

The result of second optimization at various ratios of surfactant and cosurfactant is shown in Table 4. Based on the results of both optimizations, B4 formula contained the total amount of Tween 80 and PEG 400 of 24% w/w at the ratio of 2:1 was selected as the optimum composition.

Table 4: Nanoemulsion characterization at various rasio of Tween 80 and PEG 400 with the total amount of 24% w/w

Formula	Ratio of Tween 80 to PEG 400	Visual appearance	Globule size (nm)	Stability after centrifugation
B1	1:2	Cloudy	-	-
B2	1:3	Cloudy	-	-
B3	3:1	Clear	23.07 ± 0.45	Stable
B4	2:1	Clear	21.67 ± 0.87	Stable

BSA and artin-M entrapment in nanoemulsion

In the preparation process, BSA and artin-M were made as solid dispersion form with the addition of PEG 20000, because both of them are hydrophillic that become hard to be dispersed in VCO. PEG was used due to it's good solubility in many organic solvent, including oil. High molecular weight PEG such as PEG 20000 was used because the higher molecular weight of PEG, the fewer amount of PEG will be used to obtain the same protection of protein. During freeze drying, PEG shields protein by making steric hindrance caused by preferential hidration of the protein chain in PEG matrix. Every unit of ethylen glycol in PEG can bind one molecule of water, in other word one molecule of PEG 20000 can bind 20000 molecules of water [18]. PEG 20000 also has lower toxicity compared to smaller molecular weight PEG, so it will be safer in usage [19]. Table 5 showes the characteristic of B4 nanoemulsion formula after the addition of BSA and artin-M solid dispersion. All formula showed a good performance in which possessed small globule size, good polydispersity index, and stable after centrifugation. The globule size was slightly increased from 21.67 ± 0.87 to 34.5 ± 0.79 with the existence of both hydrophilic substances. However, no particle aggregation was observed in nanoemulsion as shown in Fig. 1. It is suggested the success of PEG 20000 addition to prevent BSA and artin-M aggregation in VCO.

Table 5: Optimization and evaluation of B4 nanoemulsion formula after the addition of BSA-artin-M solid dispersion in PEG 20000

Formula	Ratio of BSA-artin-M to PEG 20000	Globule diameter (nm)	Polydispersity Index	Stability after centrifugation
C1	1:1	34.5 ± 0.79	0.341 ± 0.04	Stable
C2	1:2	37.9 ± 0.96	0.275 ± 0.01	Stable
C3	1:3	43.3 ± 3.5	0.348 ± 0.04	Stable
C4	1:4	47.37 ± 2.14	0.300 ± 0.01	Stable

Fig. 1: The appearance of nanoemulsion containing solid dispersion of BSA and artin-M (left) and water (right). Each container was irradiated by red laser light. The red light path was only observed within nanoemulsion media due to the reflection of light by The Tyndall efect of the globules.

BSA entrapment efficiency was carried out to observe the best BSA entrapment of all formula. Table 6 showes the result of BSA entrapment efficiency test of nanoemulsion. The formation of BSA solid dispersion in PEG 20000 resulted in a high BSA entrapment efficiency in the oil phase of nanoemulsion that almost reached 100%. It indicates that PEG 20000 was suitable in increasing dispersibility of BSA in VCO. In contrast, the entrapment efficiency of free BSA in oil phase was very low (6.46 ± 0.57%). It occured because BSA underwent denaturation in oil. Hydrophobic part of BSA was exposed and interacted within oil environment, hence causing unfolding and aggregation of protein molecule [20]. This protein aggregates sedimented out from the VCO globules and precipitated with addition of TCA.

In the next process, the formula C1 was chosen to be optimized further. This formula underwent BSA dosage enhancement, from 2 mg/g to 5 mg/g, and also addition of artin-M 50 µg/g. The dosage of artin-M used was 50 µg/g because it was proven in stimulating antibody production in rat [13]. The formula containing 5 mg/g BSA and 50 µg/g artin-M had average globule diameter of 42.7 ± 0.9 nm, polydispersity index of 0.321 ± 0.01, and BSA entrapment efficiency of 98.6 ± 0.15%. The nanoemulsion was also stable after centrifugation at 3750 rpm for 5 hours. The preparation also remained stable after centrifugation at 3750 rpm for 5 hours with no sedimentation or turbidity observed.

Table 6: BSA and artin-M entrapment efficiency of nanoemulsion

Ratio of BSA and PEG 20000	Entrapment efficiency (%)
1:1	98.8 ± 0.43
1:2	99.1 ± 0.15
1:3	99.1 ± 0.43
1:4	99.1 ± 0.25
BSA without PEG 20000	6.46 ± 0.57

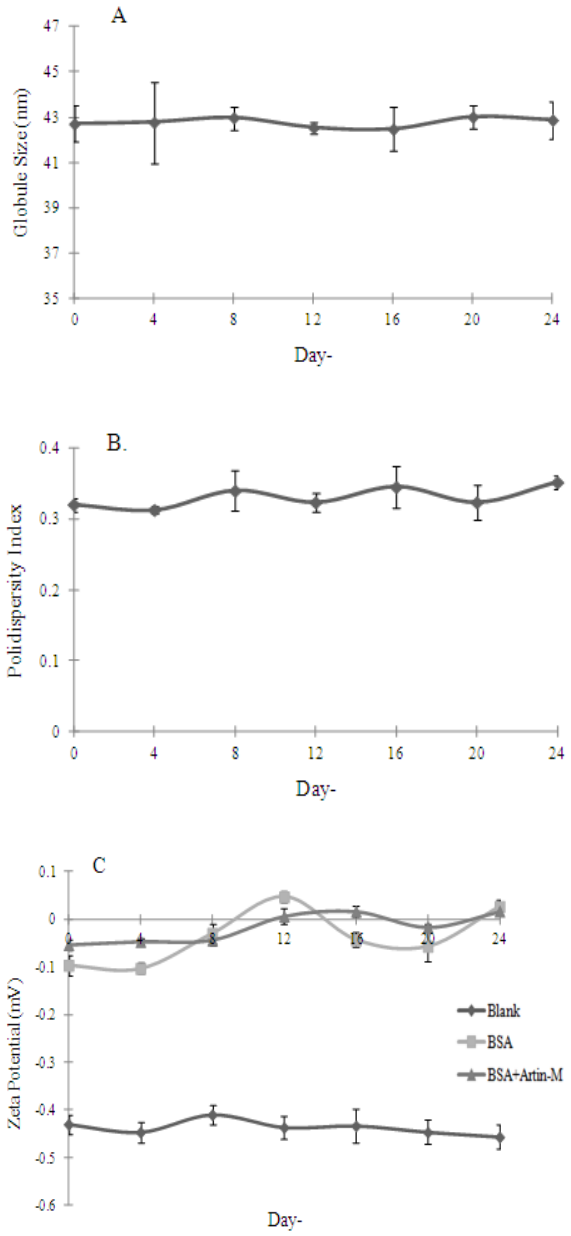

Fig.2: Nanoemulsion stability evaluated by storage of non-aqueous oil phase at freeze thaw condition until 6 cycles and reconstituted using deionized water at the end of every cycle. The nanoemulsion was characterized, including: (A) globule diameter, (B) polydispersity index, and (C) zeta potential. All parameters tested showed the stable nanoemulsion with no significant change.

Stability of nanoemulsion

To evaluate whether BSA and artin-M aggregated in the oil phase during storage, the accelerated test by freeze thaw method was conducted. The BSA-artin-M non-aqueous oil phase was kept in alternating storage at 4 °C in the fridge and room temperature for 48 hours in each condition (one cycle). The process was repeated until 6 cycles. The results of physical characteristics of reconstituted nanoemulsion analyzed at the end of each cycle are shown in Fig. 2 and Fig. 3.

Fig. 2 shows that the globule size was stable during the 24 days of freeze thaw. The average globule size was about 42 nm with polydispersity index was about 0.3 to 0.35. The size was in the range of appropriate and safe globule size (40-300 nm) for transdermal application [16]. Polydispersity index value of nanoemulsion fluctuated slightly during the time of testing, but the value was still in acceptable range. Polydispersity index describes the uniformity distribution of globules in nanoemulsion. Good polydispersity index has a value below 0.5, while the value above 0.5 indicates that the distribution of globule is non-uniform.

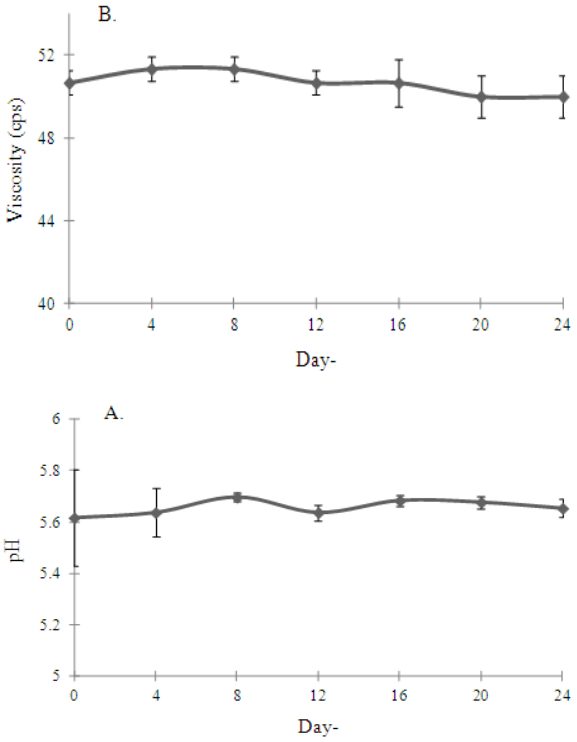

Fig. 3: Nanoemulsion stability during 6 cycles of freeze thaw and evaluated for (A) the pH and (B) the viscosity. The data show a stable nanoemulsion.

Zeta potential value of nanoemulsion containing BSA had more positive value than that of blank nanoemulsion. The zeta potential value of nanoemulsion containing BSA and artin-M was slightly fluctuated and had an average value similar to nanoemulsion containing BSA only. The change of zeta potential value was due to the positive charge of both BSA and artin-M. There was no statistical significant difference between the two zeta potential values of the nanoemulsion. It can be concluded that the addition of artin-M into the preparation did not affect the zeta potential value. Ideally, the best value of zeta potential that can disperse perfectly globules to provide electrical repulsion and prevent aggregation among the globules is above ± 30 [21]. In addition to the stabilization of electrical repulsion,

the stability of the dispersed globules is also influenced by steric stabilization [21]. Polysorbate 80 and PEG 400 used as surfactant and co-surfactant, had a role in steric stabilization of globules. Although the zeta potential value was not optimal, the existence of steric stabilization provided by Tween 80 and PEG 400 could make the globule of nanoemulsion remained stable during the testing period.

The pH value of nanoemulsion during 24 days of freeze thaw was fairly stable in the range of 5.5 – 5.8. The pH value was in the range suitable for skin preparation, which was 4.2 to 6.2. Viscosity of nanoemulsion during freezethaw was also quite stable with the value about 51 cps. The viscosity value was quite low because the nanoemulsion was of oil in water type with the amount of water 73%. This will guarantee the ease of administration.

Hemagglutination test was conducted to evaluate the activity of artin-M. This test is based on artin-M ability to bind the sugar groups on the membrane of red blood cells and cause hemagglutination [22]. Hemagglutination test was carried out on artin-M solution, solid dispersion of artin-M, and solid dispersion of artin-M in the nanoemulsion. Table 7 shows the hemagglutination activity of artin-M solution, the solution of artin-M solid dispersion in PEG 20000, and artin-M in nanoemulsion. Artin-M activity decreased to approximately 60% after its incorporation in nanoemulsion. This most likely occurred because artin-M was in the oil phase, thereby reducing contact with red blood cells. Hemagglutination test did not occur in nanoemulsion without artin-M, so it was convinced that the hemagglutination activity occurred due to the ability of artin-M to bind erytrocytes membrane and not resulted by other nanoemulsion components. Albeit the reduction of hemagglutination activity, artin-M remained active until the end of 6 cycles of freeze thaw. This result indicated that artin-M was not aggregated in the VCO globule. This statement was also supported by organoleptic observation of nanoemulsion that showed no-aggregation that did not change during the test period.

Table 7: Hemagglutination activity of Artin-M entrapped in nanoemulsion

Preparation	Minimum concentration of artin-M caused visible heemagglutination (µg/mL)			
	Day- 0	Day- 8	Day- 16	Day- 24
Aqueous solution of artin-M	1.95	1.95	1.95	1.95
Solid dispersion of artin-M	1.95	1.95	1.95	1.95
Nanoemulsionartin-M	3.12	3.12	3.12	3.12
Nanoemulsion without artin-M	-	-	-	-

CONCLUSION

Formula of nanoemulsion for administering protein vaccine with artin-M adjuvant has been successfully developed. The formula consisted of 3% VCO, 16% Tween 80, 8% PEG 400, and deionized water. BSA (5 mg/g) and artin-M (50 µg/g) were incorporated in the oil phase as co-lyophilized solid dispersion in PEG 20000 with the optimum rasio of 1:1. The success of PEG 20000 addition was shown by a high entrapment efficiency of BSA (98.6 ± 0.15%) in the oil phase of nanoemulsion and maintaining hemagglutination activity of artin-M. The nanoemulsion was spontaneously formed that resulted in the uniform globule diameter formation. The globule uniformity was maintained throughout accelerated testing period in the alternate storage at 4 °C and room temperature for the total 24 days. Both BSA and artin-M were dispersed uniformly in the oil phase during the storage with no-aggregation observed in the reconstituted nanoemulsion. These results indicate the potensial use of nanoemulsion formulation for spontaneous delivery of protein vaccine and lectin adjuvant through non-invasive transdermal administration.

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