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RESEARCH ARTICLE

Vitamin E-based Folic Acid Nanoemulsion: Formulation and Physical Evaluation for Oral Administration

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Abstract: ***Background*:** Folic acid is essential in many metabolic processes and DNA synthesis. Nevertheless, folic acid is not stable, pH-sensitive, and deteriorated upon light exposure.

***Objective*:** This work was aimed to improve folic acid stability within vitamin E-based nanoemulsion.

***Methods*:** The nanoemulsion was prepared with self-nanoemulsification method by mixing vitamin E oil, Tween 20, and PEG 400. A pseudoternary phase diagram was constructed with aqueous titration to determine the optimum ratio for the mixture. The globule size, pH and entrapment efficiency were included in the nanoemulsion characterizations. In addition, the influence of centrifugation, storage, and pH on physical and chemical stabilities of folic acid nanoemulsion was evaluated.

***Results*:** Optimum formula was obtained from vitamin E, Tween 20, and PEG 400 with the ratio of 1:11:1, and the folic acid amount was 8 mg. The size of folic acid-loaded oil globule was 15.10 ± 1.51 nm, and the nanoemulsion pH was 6.24 ± 0.01. The nanoemulsion system was able to load the folic acid completely. Folic acid in nanoemulsion was stable after 14 days at room temperature, and it was more stable compared to folic acid in solution. In addition, the physical and chemical characteristics of folic acid in nanoemulsion was not affected by the simulated gastric condition.

***Conclusion*:** Hence, nanoemulsion is a promising strategy to enhance folic acid stability.

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1. INTRODUCTION

Folic acid has a vital role in the cellular growth and development, mainly in its ionic form. In the body, folate (the anionic form of folic acid) acts as a cofactor for many important cellular reactions including the transfer of single-carbon units, which is required for cell division in the DNA synthesis [1]. Thus, folate deficiency may cause abnormal cell division due to DNA synthesis interference. One of the first known clinical manifestations of folate deficiency is hypersegmentation of the neutrophils [2].

Supplementation of folic acid has been recommended for pregnant women since 1991 [3]. The recommendation was based on findings that folic acid consumption can prevent neural tube defects, which are among the most common severe congenital malformations [3]. In elderly people, folic acid deficiency has been associated with neuro-

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psychiatric disorders in the absence of anemia, for instance in some depressions and dementias, including Alzheimer's disease [4].

In commercial products, folic acid is often available in combinations with other vitamins. Its most stable storage condition is in aqueous solutions at a pH of 5-8 [5, 6]. Folic acid is well-known to be photolabile, because of its capability in absorbing UV irradiation [1, 7, 8]. The molecular structure of folic acid is divided into three parts: a glutamic acid moiety, a p-aminobenzoic acid moiety, and a pterin moiety [1, 9]. Upon exposure to UV light, folic acid is degraded by cleavage on the methylene bridge into 6-formylpterin and p-aminobenzoyl-L-glutamic acid. Further UV irradiation led to cleavage of p-aminobenzoyl-L-glutamic acid leading to p-aminobenzoic and glutamic or degradation of 6-formylpterin to pterin-6-carboxylic acid [1, 7, 10, 11]. At temperatures lower than 180°C, folic acid is relatively stable because glutamic acid, pterin and p-aminobenzoic are degraded at 180°C [1].

In this research,the stability of folic acid was improved by formulating folic acid in nanoemulsion system. Folic acid was incorporated in the system stabilized by the mixture of surfactant and cosurfactant. Thus, folic acid was expected to be stable in the aqueous dispersion system. Self-nanoemulsification (SNE) or spontaneous method was applied to produce nanoemulsion which can further be incorporated in the liquid dosage form. It is known that oral liquid dosage form offers several advantages, *i.e*. increased drug bioavailability, better patient compliance, and flexibility in dosing especially in situations where dose adjustments in hepatic or renal impaired patients are required [12]. Most importantly, it is ideal for patients who cannot swallow tablets or capsules. Nanoemulsion can be formed spontaneously when there is less required free energy. The presence of globule interfacial stabilization decreases the surface tension, so the required free energy for the nanoemulsion production is very low [13].

2. MATERIALS AND METHODS

2.1. Materials

Folic acid (PT Kimia Farma, Bandung, Indonesia), vitamin E (PT Kimia Farma, Bandung, Indonesia), Montanox 20 DF (Tween 20, PT Megasetia Agung Kimia, Indonesia), polyethylene glycol 400 (PEG 400, Merck, Darmstadt, Germany), sodium carbonate (Merck, Darmstadt, Germany), methanol, acetate buffer pH 5.7, acetonitrile pro analysis (J.T. Baker), hydrochloride acid, sterile aquadest (IKA, Indonesia), deionized water (Bandung Institute of Technology, Indonesia).

2.2. Nanoemulsion Preparation

SNE method was applied in the formulation development, in which vitamin E, Tween 20, and PEG 400 were used as oil phase, surfactant, and cosurfactant, respectively. The oil mixture was produced by mixing vitamin E, Tween 20, and PEG 400 using magnetic stirrer for 2 hours and sonicated for 60 minutes. To produce nanoemulsion, deionized water was added to the oil mixture dropwise (oil phase: aqueous phase = 1:4), and the mixture was stirred gently with a magnetic stirrer until a clear system was obtained. Globule size analysis and visual appearance were used to evaluate the optimized formula.

2.3. Pseudoternary Phase Diagram

A pseudo ternary phase diagram of the nanoemulsion was determined by mixing vitamin E with the mixture of Tween 20 and PEG 400 (11:1). The ratio of the mixture was varied from 1:9 to 9:1. The mixing was carried out by using magnetic stirrer, and deionized water was titrated into the mixture. The visual appearance of the mixture was observed, and the specific ratio resulted in nanoemulsion system was used to generate the diagram.

2.4. Folic Acid-loaded Nanoemulsion

The optimization on the folic acid entrapment in the nanoemulsion system was carried out by varying the amount of folic acid (2, 4, 6, 8, and 10 mg) added into 10 g of oil mixture. The mixing procedure was done by using magnetic stirrer for 2 hours and sonicated for 60 minutes. After that, deionized water was added into the mixture with the oil-water ratio of 1:4, and the mixture was stirred to produce folic acid nanoemulsion system. The evaluations on the obtained nanoemulsion included visual appearance, globule size, and entrapment efficiency.

2.5. Physical Evaluation of Folic Acid-loaded Nanoemulsion

2.5.1. Globule Size and Polydispersity Index

Globule size and polydispersity index were determined with photon correlation spectroscopic method by using particle analyzer (Delsa™ Nano C Particle Analyzer, Beckman Coulter). The sample was analyzed at 25°C.

2.5.1.1. Entrapment Efficiency Determination

The entrapment efficiency of folic acid in the nanoemulsion system was determined with a direct method by comparing the entrapped folic acid in the globule with total folic acid in the system. The entrapped and free folic acid was separated by centrifugation of folic acid nanoemulsion at 13,000 rpm for 20 minutes. The free folic acid precipitated, while the entrapped folic acid was in the supernatant.

The entrapped folic acid was determined by using High-Performance Liquid Chromatography (HPLC, Agilent) with C-18 column (Phenomenex® Luna C_{18}, 5 µm, 100 Å, 250 x 4.6 mm) and UV-visible detector at 365 nm. Sodium acetate buffer (0.1 M, pH 5.7) and acetonitrile were used as mobile phase with the ratio of 94:6 and flow rate of 1 mL/min. The sample was first mixed with methanol (1:1) by using vortex for 30 seconds. After that, 600 µl of 0.01 M sodium carbonate was added into the mixture and mixed with vortex for 60 seconds. Total folic acid was determined by using a similar procedure without prior centrifugation. The entrapment efficiency was calculated using the following equation:

Entrapment efficiency = [Entrapped folic acid /Total folic acid] x 100%

2.5.2. pH Determination

Nanoemulsion pH was determined by using a calibrated pH meter (Mettler Toledo® S20).

2.6. Stability Test

2.6.1. Centrifugation Test

Folic acid nanoemulsion was centrifuged to evaluate its physical stability. The centrifugation was carried out at 13,000 rpm for 30 min, and the effect on the visual appearance of nanoemulsion was evaluated. Folic acid formulated in conventional emulsion was used as control.

2.6.2. Stability During Storage

The stability of folic acid nanoemulsion after 14 days at room temperature was analyzed. The observations included globule size, polydispersity index, entrapment efficiency, and pH.

2.6.3. Stability at pH 1.2

The physicochemical alteration of folic acid nanoemulsion in the simulated gastric condition was evaluated *in vitro* by using HCl solution pH 1.2. As a comparison, folic acid in 2 g of oil phase containing vitamin E, Tween 20 and PEG 400, was mixed with 8 mL of HCl solution pH 1.2. Globule size, size distribution, and folic acid content were parameters evaluated in this study.

3. RESULTS AND DISCUSSION

Nanoemulsion formula was developed by varying the ratio of vitamin E oil, Tween 20, and PEG 400. The use of vitamin E as oil phase has beneficial effects for the system. Besides its ability to dissolve folic acid, vitamin E has an antioxidant property that can protect folic acid from oxidation.

Nanoemulsion formation requires high free energy because it has a very large surface area to maintain the globule dispersion. Tween 20 is a surfactant with both hydrophilic and hydrophobic properties, which can form an interfacial layer between water and oil. This layer can decrease the water-oil interfacial tension, which allows the formation of nanoemulsion. A surfactant that can be used to produce oil in water nanoemulsion must have hydrophylic lipophilic balance (HLB) above 10. Tween 20 has HLB of 16.7, so it can be used as a surfactant to produce nanoemulsion. Tween 20 is also a nonionic surfactant with relatively low toxicity [14].

In addition to Tween 20, PEG 400 was used as a cosurfactant to complete the function of Tween 20 in reducing the water-oil interfacial tension. PEG 400 has two hydroxyl and relatively short ethylene groups. These groups are able to penetrate the interfacial area and form a compact layer of surfactant-cosurfactant [15].

Table 1. The characteristics of folic acid-loaded nanoemulsion with various compositions of oil-surfactant-cosurfactant.

Formula	Oil ratio	Surfactant Ratio	Cosurfactant Ratio	Visual Appearance	Globule Size*(nm)	Polydispersity Index
F1	1	7	1	Cloudy	457.30 ± 47.98	0.30 ± 0.02
F2	1	8	1	Slightly cloudy	91.73 ± 13.77	0.30 ± 0.03
F3	1	9	1	Slightly cloudy	35.63 ± 1.53	0.28 ± 0.03
F4	1	10	1	Clear	18.47 ± 5.07	0.27 ± 0.11
F5	1	11	1	Clear	14.20 ± 1.28	0.20 ± 0.13
F6	1	7	2	Cloudy	357.88 ± 112.15	0.31 ± 0.04
F7	1	8	2	Slightly cloudy	90.20 ± 4.33	0.36 ± 0.03
F8	1	9	2	Slightly cloudy	20.17 ± 0.75	0.32 ± 0.01
F9	1	10	2	Clear	16.00 ± 1.38	0.27 ± 0.02
F10	1	11	2	Clear	17.67 ± 4.65	0.31 ± 0.05

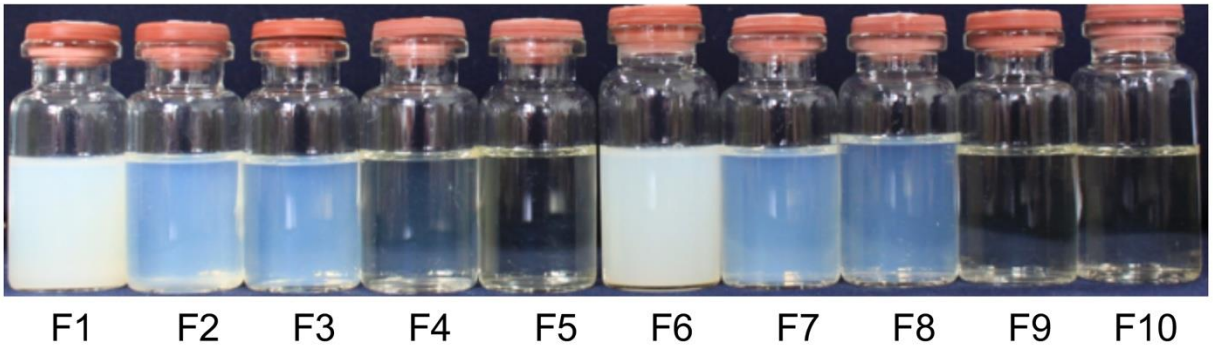

Fig. (1). Visual appearance of nanoemulsion formula F1 to F10 (left to right).

Among formulas evaluated in this study, formula F5 generated the optimum characteristic of nanoemulsion prototype with oil, surfactant, and cosurfactant with the ratio of 1:11:1 (Table **1**) (Fig **1**). This composition produced a clear and transparent appearance of nanoemulsion with the smallest globule size of 14.20 ± 1.28 nm and narrow size distribution. The high concentration of surfactant was required to generate spontaneous nanoemulsion with SNE method. This is due to the structure of vitamin E that consists of chromanol ring and long alkyl side chain, causing the hydrophobic property of vitamin E. Oil with long hydrocarbon chain is relatively harder to be emulsified compared to oil with medium or short chain [16, 17].

Based on the optimization result, surfactant-cosurfactant ratio (11:1) was chosen as a component in generating the diagram (Fig. **2**). Monophase area indicates the nanoemulsion formation, in which the obtained system is clear and transparent [18].

As shown in Fig. (**2**), nanoemulsion was only formed in the specific condition of oil phase, surfactant-cosurfactant and water. In this specific condition, the required free energy to generate spontaneous nanoemulsification is lower [19, 20]. In addition, vitamin E concentration was considered low with a large area of Tween 20-PEG 400 (Fig. **2**).

The specific ratio component of nanoemulsion was used to optimize the added amount of folic acid in the system. Different amount of folic acid was added into the mixture of vitamin, Tween 20, and PEG 400 (1:11:1).

Globule size and size distribution are important characteristics in the nanoemulsion system, which

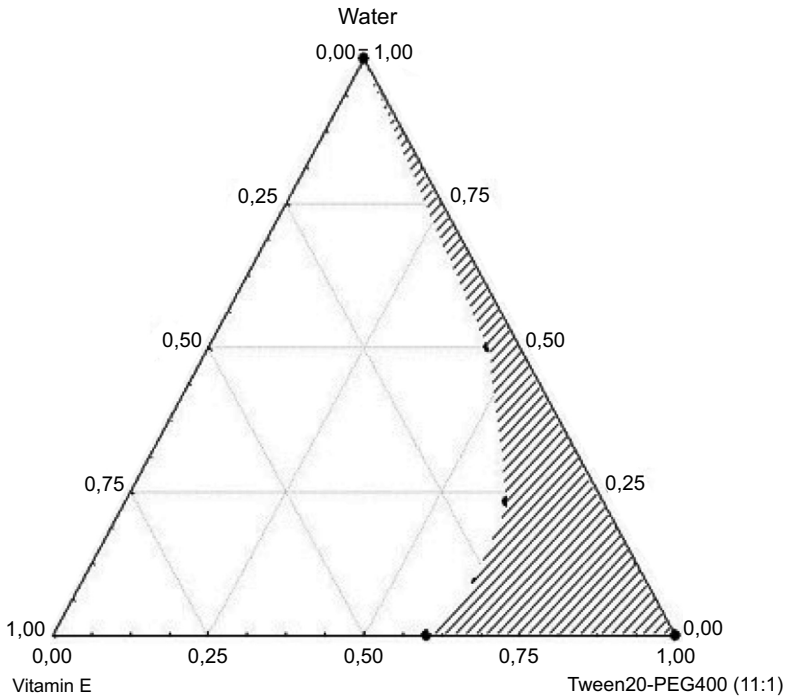

Fig. (2). Pseudoternary diagram of nanoemulsion. The shaded area showed the composition forming nanoemulsion.

Table 2. Globule size and polydispersity index of folic acid nanoemulsion.

Formula	Folic Acid in 10 g Oil Phase (mg)	Visual Appearance	Globule Size (nm)	Polydispersity Index
A	2	Clear	14.17 ± 0.40	0.21 ± 0.07
B	4	Clear	14.70 ± 1.78	0.20 ± 0.08
C	6	Clear	14.93 ± 2.52	0.13 ± 0.06
D	8	Clear	15.10 ± 1.51	0.31 ± 0.06
E	10	Slightly cloudy	118.10 ± 3.29	0.34 ± 0.01

determine the *in vivo* drug distribution, toxicity, and controlled release capability. On top of that, globule size and size distribution influence drug entrapment, drug release profile, nanoemulsion stability [21]. Particle size analysis of folic acid nanoemulsion after mixing oil phase with deionized water is shown in Table **2**.

As given in Table **2**, the globule size increased as the amount of folic acid increased. Clear nanoemulsion system was obtained from formula A, B, C, D with folic acid content ranging from 2 to 8 mg (Fig. **3**). When 10 mg of folic acid was incorporated, the globule size increased significantly to 118.10 nm, and the system was slightly cloudy. This can be explained from the limited solubility of folic acid in the oil phase. Drug solubility in oil determines its entrapment in the nanoemulsion system and the characteristics of the nanoemulsion, especially particle size [22].

Polydispersity index indicates the homogeneity of nanoemulsion globule size. The homogenous system has a polydispersity index close to 0 [23]. Formula A to E had a polydispersity index from 0.13 to 0.34, which indicated homogenous size distribution (Table **2**).

The entrapment efficiency of folic acid in the nanoemulsion system was determined with the direct method, in which entrapped folic acid was measured and compared to total folic acid in the dosage form. To reach the highest entrapment efficiency, the drug must interact with the carrier system and not the medium [24]. Thus, it has to be

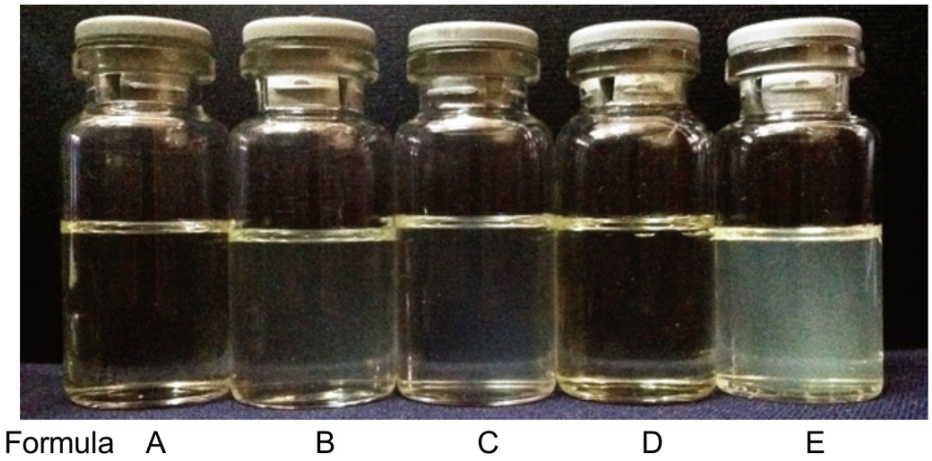

Fig. (3). Visual appearance of folic acid nanoemulsion formula A, B, C, D, and E, containing 2, 4, 6, 8, and 10 mg folic acid respectively (left to right).

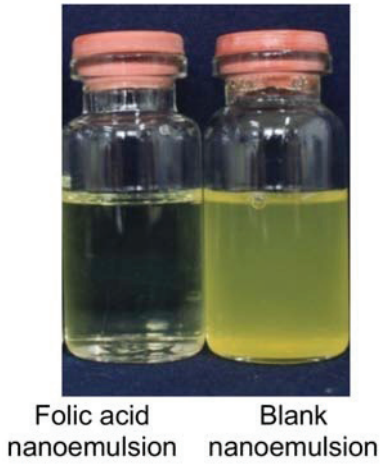

Fig. (4). Visual appearance of folic acid nanoemulsion (left) and blank nanoemulsion with the addition of folic acid (right).

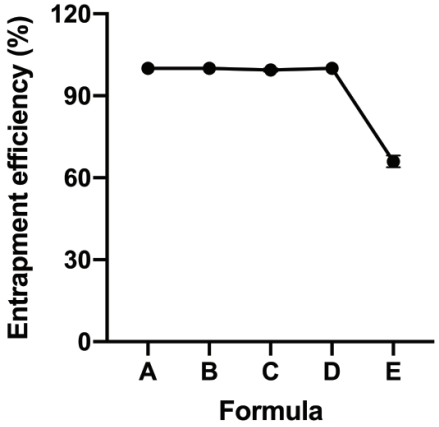

Fig. (5). Influence of folic acid amount on the entrapment efficiency of folic acid in nanoemulsion. Formula A, B, C, D, and E used 2, 4, 6, 8, and 10 mg of folic acid, respectively.

proven that folic acid is entrapped in the nanoemulsion globule and not dissolved in the water.

Fig. (**4**) shows the comparison of folic acid in the nanoemulsion (entrapped in oil globules) and freely dispersed in the aqueous medium of blank nanoemulsion systems. As seen, folic acid-loaded nanoemulsion has clear and transparent appearance (left) as compared to blank nanoemulsion added with folic acid (right). This proves that additional folic acid in the external medium was not entrapped in the oil globule. On the contrary, the clear appearance of folic acid nanoemulsion indicates entrapped folic acid in the oil globules. If the system is centrifuged, free folic acid will precipitate but entrapped folic acid will stay in the oil globules.

The influence of incorporated folic acid on entrapment efficiency is shown in Fig. (**5**). The entrapment efficiency of folic acid in the nanoemulsion system with 2 to 8 mg of folic acid was almost 100%. However, it decreased significantly to 66% when 10 mg of folic acid was incorporated. The physicochemical properties and interaction of folic acid with other components in the system might be an explanation. The limited solubility of folic acid in the oil phase causes the available amount of vitamin E was not able to dissolve the incorporated folic acid. Above its maximum capacity, the oil globules in the nanoemulsion system cannot entrap all incorporated folic acid. So, folic acid was only dispersed outside the globules. Entrapment efficiency will decrease when the amount of incorporated drug is above the maximum capacity of the nanoemulsion system.

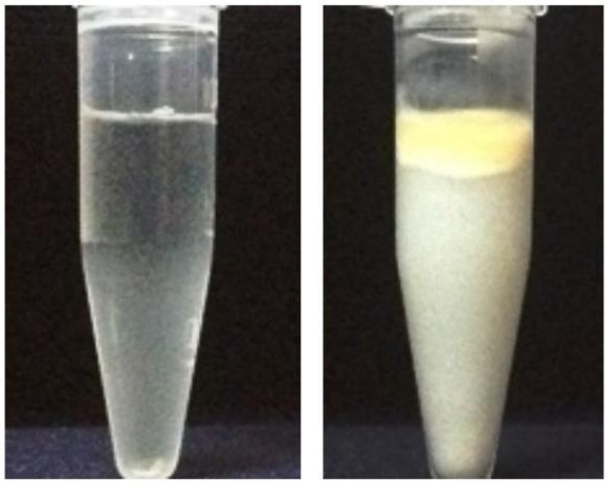

Fig. (6). The physical appearance of folic acid nanoemulsion (left) and conventional emulsion (right) after high speed centrifugation.

Based on the results given in Table **2** and Fig. (**5**), formula D with 8 mg of folic acid incorporated into 10 g of the oil phase was chosen to be further evaluated. Formula D showed clear and transparent nanoemulsion with particle size < 50 nm, homogeneous size distribution, entrapment efficiency of 100%, and pH of 6.24 ± 0.01. The pH of the system is critical for the chemical stability of folic acid. In general, folic acid is stable in a medium with pH 5 to 8 [5]. Thus, formula D is in the stable pH range of folic acid.

To predict the physical stability of nanoemulsion, the system was challenged by high-speed centrifugation. According to Stokes' Law, nanoemulsion centrifugation can increase the globule migration rate towards the superficial layer of the nanoemulsion system. As a consequence, there is an increased chance of globule aggregation, which can destroy the surfactant monolayer and form coagulation [25]. The resistance of the nanoemulsion against high-speed centrifugation is shown in Fig. (**6**). On the contrary, folic acid in the conventional emulsion system showed separation into two phases. As nanoemulsion used surfactant and cosurfactant in a relatively higher amount compared to the conventional emulsion, thus the globules in nanoemulsion are more stable. The presence of cosurfactant optimized the function of surfactant to protect the globules from aggregation.

Fig. (**7**) shows the stability profile of folic acid nanoemulsion after 14 days of storage. As seen, the nanoemulsion stayed in the range of 15.0-16.0 nm with a polydispersity index of 0.16-0.31. Furthermore, the system pH was in the range of 5.9-6.4 with the entrapment efficiency of folic acid in the range of 92-100%. There was no significant change of globule size as well as the entrapment efficiency after 14 days of storage at room temperature. This indicated that the system maintained the medium pH, which created a stable condition for folic acid.

To confirm the stability data as presented in Fig. (**7**), the monitoring of folic acid content was also performed during the stability study. For this purpose, the amount of folic acid in nanoemulsion was determined after 14 days of storage. Folic acid in sodium carbonate solution was used as control.

While folic acid content in nanoemulsion was decreased to 86% after 14 days of storage, folic acid content in sodium carbonate solution decreased to 31% (Fig. **8**). It means that folic acid in nanoemulsion was 2.7 times significantly higher compared to folic acid in sodium carbonate solution after 14 days of storage ($p<0.05$). This indicates that folic acid in nanoemulsion has better stability during storage.

In the nanoemulsion system, folic acid is entrapped in the oil globules. The system stabilization was formed by surfactant and cosurfactant on the interface of globules, which protected folic acid from medium and external factors. The use of

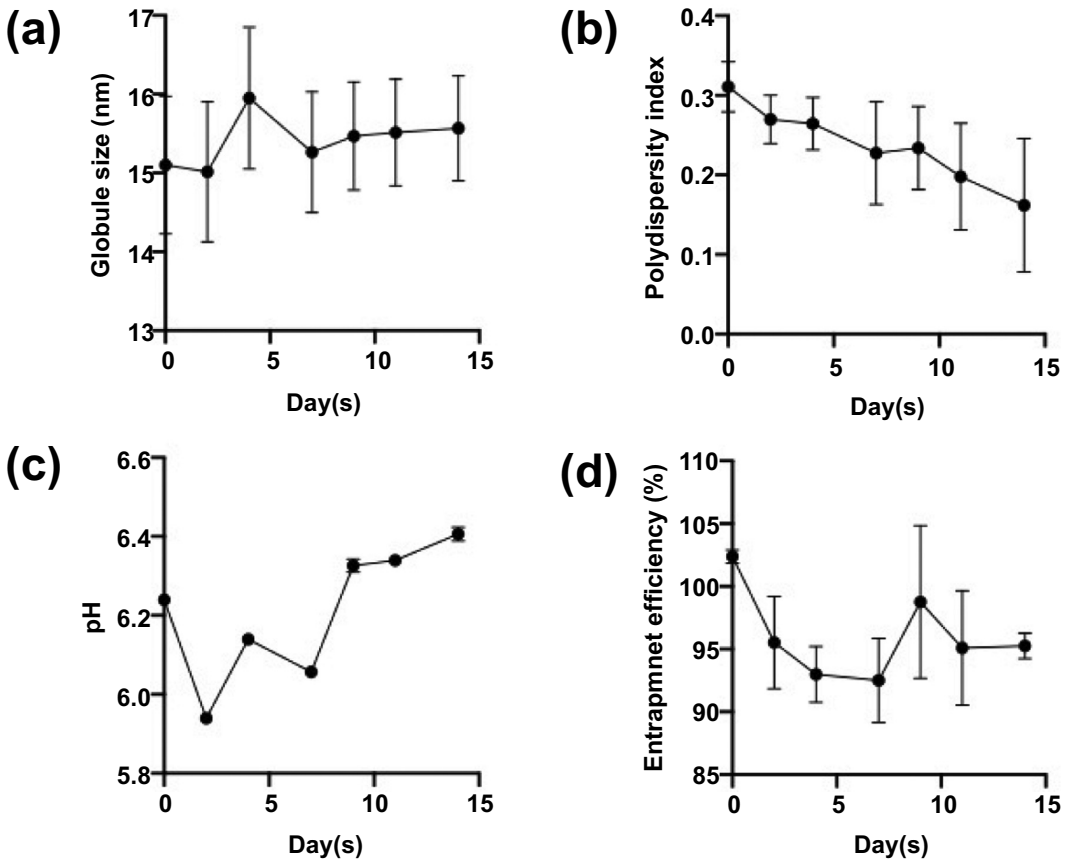

Fig. (7). Folic acid nanoemulsion stability at room temperature after 14 days, which included globule size (**a**), polydispersity index (**b**), pH (**c**), and entrapment efficiency (**d**).

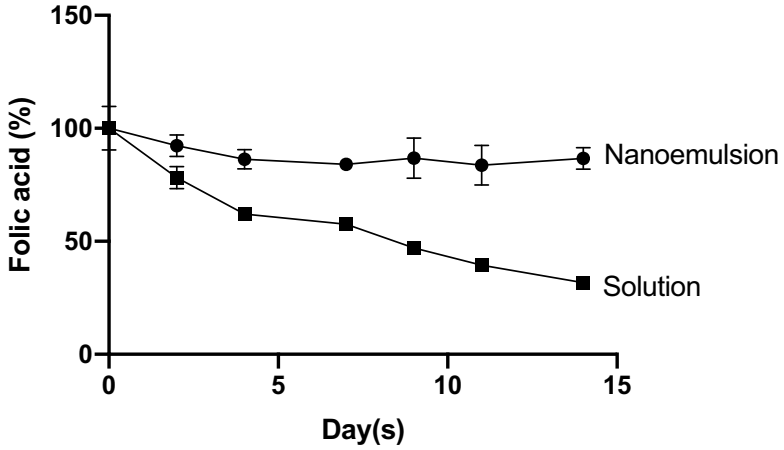

Fig. (8). Influence of storage on the content of folic acid in nanoemulsion (•) and solution (■) at room temperature.

Table 3. Influence of HCl pH 1.2 on physical and chemical properties of folic acid nanoemulsion.

External Phase	Globule Size (nm)	Polydispersity Index	Folic Acid Content (ppm)
Deionized water	15.10 ± 1.51	0.31 ± 0.05	123.11 ± 1.70
HCl pH 1.2	17.03 ± 3.31	0.30 ± 0.13	118.21 ± 8.30

vitamin E as the oil phase also acts as an antioxidant that can protect folic acid from oxidation.

In addition to the data shown in Fig. (**8**), the stability test was also done in HCl pH 1.2, a simulated gastric acid fluid. As described in Table **3**, there was no significant difference of globule size, polydispersity index, and folic acid content in the nanoemulsion system with deionized water and HCl pH 1.2 as an external medium.

4. CURRENT AND FUTURE DEVELOPMENTS

Folic acid is one of the most essential supplements that has been recommended for pregnant women since 1991. However, it is known that folic acid is easily degraded and very sensitive to UV light, heat, and oxygen. Folic acid is commercially available alone or in combinations with other vitamins as solid and liquid dosage forms. Although the formula development of oral liquid dosage form is more challenging, it is more preferable in a certain condition such as dose adjustment for hepatic or renal impaired patients. In addition, liquid dosage form has higher bioavailability than solid dosage form. Nanoemulsion system offers improved stability because it has high kinetic stability and can protect folic acid from extreme conditions. In addition, the nanoemulsion system improved the bioavailability of several compounds. One of them was curcumin, which was developed in our laboratory. Our current study showed that folic acid nanoemulsion is physically and chemically more stable than conventional emulsion and solution. In the future, an accelerated stability test will be done to prove that our formula is stable in longer-term for commercial purposes.

CONCLUSION

The optimum formula for folic acid in this study was obtained using vitamin E, Tween 20, and PEG 400 with the ratio 1:11:1, and 8 mg of folic acid per 10 g of oil phase mixture. This formula generated clear and transparent nanoemulsion with globule size of 15.10 ± 1.51 nm, polydispersity index of 0.31 ± 0.06, pH of 6.24 ± 0.01 and entrapment efficiency of 100%. Folic acid nanoemulsion was stable after centrifugation, stored in room temperature for 14 days, and it was physically and chemically stable in simulated gastric acid condition pH 1.2. Nanoemulsion system has been proven to increase folic acid stability and decrease the degradation rate of folic acid in water medium significantly compared to folic acid in solution.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [Dr. Amirah Adlia], upon request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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