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Development of Ethosomes Containing Mycophenolic Acid

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Abstract

Mycophenolic acid (MPA) is mainly considered an immunosuppressive drug because it is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), and selectively inhibits T- and B-cell proliferation. The aim of this study was to develop the ethosome containing MPA to enhance skin permeation after topical administration for the enhancement of skin permeation after topical administration. In this study, the analysis of MPA was performed using HPLC method. The effects of pH and ethanol contents on MPA solubility were evaluated to find out the suitable dispersion medium for the ethosome preparations. The solubility profiles of MPA were investigated as a function of pH and ethanol content. In addition, the effects of formulation compositions on the physical appearance, entrapment efficiency, zeta potential, particle size and size distribution were investigated. It was found that the ethosome of MPA (10 mg/mL) could be prepared by using the thin film hydration method. The optimized formulation composed of 4% w/v SPC:CHOL:Tween80:DA (6:2:1:1 molar ratio) as lipid component and 30% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium. The MPA ethosome gave the vesicular size of 371 ± 8 nm (PI = 0.27 ± 0.02 nm), the zeta potential of -46 ± 5 mV and the entrapment efficiency of 56 ± 1%.

Keywords: Mycophenolic acid; ethosome; immunosuppressive drug; inosine monophosphate dehydrogenase (IMPDH).

1. Introduction

Mycophenolic acid (MPA) is a non-competitive, selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) type II, a key enzyme in the *de novo* synthesis of guanine nucleotides within activated T and B lymphocytes and macrophages leading to an inhibition of cell proliferation. MPA has been used widely as an immunosuppressant in organ transplantation and in a variety of autoimmune diseases such as scleroderma, systemic lupus erythematosus and psoriasis [1]. MPA was used as a systemic agent to treat psoriasis in the 1970s and it was found that oral administration

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of MPA was effective in treating psoriasis. However, its oral administration has disadvantages due to extensive first-pass metabolism by the liver and its tolerability was limited by gastrointestinal (GI) side effects; it caused nausea, diarrhea, soft stools, anorexia, abdominal cramps and frequent stools which lead to most patients requiring dose changes or discontinuation of treatment [2]. It is assumed that the topical administration can be considered as an alternative route to improve these disadvantages and to avoid the GI side effects. However, the insufficiency of skin permeation of MPA formulations leads to low clinical efficiency of topical administration [3].

Ethosome carriers were first reported by Touitou *et al.* [4] and are a modified form of liposome. Ethosomes are vesicle lipid carriers containing phospholipid, and have a high concentration of ethanol and water. It is an interesting system which has important features related to ability to increase skin permeability [4]. The size of ethosomes ranges from tens of nanometers to microns which have a small size related to liposome [5]. The ethosome has an ability to efficiently entrap various molecules, like hydrophilic, lipophilic and amphiphilic molecules. It can be explained by the high degree of lamellarity and the presence of ethanol in the vesicles, which allows for better solubility of many drugs. Moreover, ethosomal formulations possess greater entrapment capability than liposome [4]. One of the important characteristics of ethosomes is due to their soft and flexible nature, they can penetrate the skin and allow enhanced delivery of various active agents to deeper strata of the skin or enhanced systemic circulation compared to conventional liposome or hydroethanolic solutions [6]. The better permeability of ethosome carriers may be due to the synergistic mechanism between high concentration of ethanol, phospholipids vesicles and skin lipids. Ethanol interacts with lipid molecules in the polar head group region resulting in increasing fluidity and may finally lead to increase membrane permeability. In addition, it may provide the vesicles with soft flexible characteristics which are easy to penetrate into deeper layers of the skin. The release of drug could be the result of fusion of ethosome with skin lipids and drug release at various points along the penetration pathway [4]. Many studies have used ethosome as a carrier system of drugs delivery for the transdermal or topical administration. It has been reported to improve various drug deliveries both *in vivo* and *in vitro* skin, such as Testosterone, Cannabidiol, Buspirone hydrochloride, Erythromycin, Ammonium glycyrrhizinate, Lamivudine and 5-aminolevulinic acid [5-8]. In this study, the ethosome containing MPA was prepared and characterized to obtain the optimized formulation which may suit for application as topical delivery system for MPA.

2. Experiment

2.1. Materials

Mycophenolic acid (MPA) was kindly supplied by the Molecular Pharmaceutical Research Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. It was synthesized by hydrolysis of mycophenolate mofetil (MMF), which was obtained from the extraction of Cellcept® capsule and identified by using chromatographic and spectroscopic techniques (MS and H-NMR) compared to the authentic MPA. L-α-phosphatidylcholine from soybean (≥30%, SPC) and stearylamine (SA) were purchased from Sigma-Aldrich. Cholesterol from lanolin (CHOL), deoxycholic acid (DA) and triethylamine were also purchased from Fluka. Triton X-100 was purchased from Baker analyzed (New Jersey, USA). Polyoxyethylene sorbitan monooleate (Tween80) was obtained from Srichand United Dispensary Co., Ltd (Bangkok, Thailand). All other reagents used in the study were of analytical grade.

2.2. Quantitative analysis of MPA using high-performance liquid chromatography (HPLC)

The HPLC method for quantitative determination of MPA used throughout this study was modified from the method described by Gopalakrishnan *et al.* [9]. The chromatographic analysis was carried out with a reverse phase BDS HYPERSIL C18 column (250 × 4.6 mm, 5 μm) at room temperature. The mobile phase was a mixture of methanol/acetonitrile/phosphate buffer (10:30:60 v/v) that was filtered through 0.45 μm nylon membrane filter and degassed by sonication prior to use. The flow rate was maintained at 1.0 mL/min. The 20 μL sample solution was injected and the absorbance was detected at 254 nm. The assay validation was performed include specificity, linearity, limit of detection and quantitation, accuracy and precision for inter-day and intra-day.

2.3. Solubility studies of MPA

The effects of pH and ethanol content on MPA solubility were evaluated to find out the suitable dispersion medium for the preparations of ethosome containing MPA. The study was conducted by adding an excess amount of MPA to glass vials containing 10 ml of the hydroethanolic solution with 0-50% v/v ethanol and the desired media which include the buffer solutions pH 1.5, 2.0, 4.5, 5.5, 6.5, 7.0, 7.4 and 8.0 that were prepared according to USP 30. The samples were sonicated for 30 minutes and observed at room temperature for 48 h and 72 h to ensure that the solubility of MPA reached equilibrium. The excess drug was filtered through 0.45 μm syringe filter membrane and the solubility of MPA in the samples was determined using HPLC at 254 nm wavelength. All determinations were performed in triplicate. For statistical analysis, the paired *t*-test was used to evaluate differences in the solubility of MPA between 48 and 72 h. A value of $p < 0.05$ was considered to be significant.

2.4. Formulation and preparation of ethosome containing MPA

The formulation and preparation of ethosome containing MPA were prepared according to the thin-film hydration method, using the concentration of MPA 10 mg/mL. The main composition of ethosome includes of 2-6% w/v SPC and hydroethanolic solution which is a mixture of phosphate buffer pH 7.4 with 10-50% v/v ethanol. In addition, additives such as CHOL, Tween80, PEG and surface charge agent (SA and DA) improve the properties of ethosome. All the components of ethosome were varied in terms of type, concentration and ratio. In comparison, the corresponding liposome containing MPA was also prepared using the same materials and procedure to describe for preparing ethosome, but without ethanol. All formulations were prepared in triplicate.

2.5. Physical appearances, particle size distribution and zeta potential analyzer

The physical appearances of all prepared ethosome were observed in terms of color, phase separation and precipitation. The particle size, size distribution and zeta potential of both liposome and ethosome suspension was determined by Zeta Potential Analyzer model Zeta PALS (Brookhaven Instruments Co., NY, USA) at 25 °C with a scattering angle (θ) of 90 degrees after diluted of 10 μL ethosome with 4 mL Milli-Q water. All determinations were performed in triplicate.

2.6. Determination of the MPA entrapment efficiency

The MPA entrapment efficiency of ethosome or liposome formulations was determined by ultracentrifuge technique. Both formulations were centrifuged at 60,000 rpm, 4 °C for 2 h using an

Optima™ L-100XP Ultracentrifuge (Beckman Coulter Inc., CA, USA) and the supernatant was collected for determination of free or non-entrapped drug using HPLC. For the total drug, the vesicles were also lysed with 30% v/v Triton X100 and diluted with mobile phase to estimate the total drug amount by HPLC. The MPA entrapment efficiency was calculated from the following formulation:

$$\% \text{ Entrapment efficiency} = \frac{T - F}{T} \times 100$$

Where; T is the total drug amount in the formulation
F is the non-entrapped drug amount in the formulation.

3. Results and Discussion

3.1. Quantitative analysis of MPA using high-performance liquid chromatography (HPLC)

In this study, MPA was quantified using HPLC. The typical chromatogram of MPA solution is showed in Figure 1. The retention time of MPA was about 5.5 min. For the method validation of the MPA assay, the linearity was determined by plotting the peak area ratio of MPA over the concentration range of 3-30 µg/mL as a standard calibration curve of MPA, which the correlation coefficients (R^2) was 0.9998. The limit of detection (LOD) and the limit of quantification (LOQ) of MPA were 0.1 and 0.3 µg/mL, respectively. The intra-day reproducibility of this assay showed that the relative standard deviation (RSD) varied between 1.5-2.7%. And the accuracy was reported in term of %recovery varied between 97-102%. The inter-day RSD varied between 1.2-2.5% and the recovery was in the range of 98-102%. These values were within acceptable limits of AOAC (1993) for both %recovery (80-110%) and %RSD (less than 7.3%). The results indicated that the MPA assay was accurate and reproducible enough for its application.

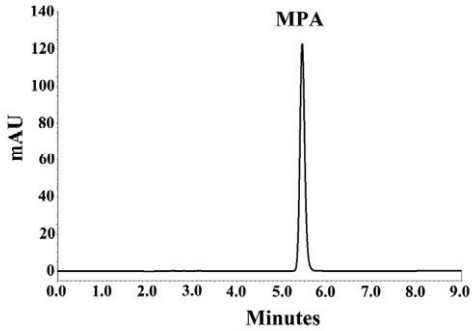

Fig. 1. A typical chromatogram from MPA

3.2. Effect of pH and ethanol on solubility of MPA

MPA is very slightly soluble in water (13 µg/mL at 25 °C), because it is a lipophilic drug. However, the solubility properties have influenced on formulation and preparation of ethosome containing MPA. We need to find an appropriate dispersion medium that is able to avoid drug precipitation in the formulation. In this case, the effects of pH on MPA solubility were evaluated. Figure 2a shows its pH-solubility profile. Since MPA is a weak organic acid with greater solubility in alkaline than in acidic medium (pK_a = 4.5) and its solubility increases as pH increases. At pH 7.0, 7.4 and 8.0, the solubility of

MPA increase to about 4.1, 5.7 and 7.3 mg/mL, respectively. Although, MPA is not stable in buffer solutions pH 8 due to it was hydrolyzed by base-catalyzed. Therefore, the buffer solutions pH 7.4 was selected for use in preparing ethosome formulation. The solubility profile of MPA in various ethanol concentrations showed that the solubility of MPA increased as ethanol concentration increased, Figure 2b. As the concentration of ethanol increased from 30, 40 and 50% v/v, the solubility of MPA increased to 0.1, 2.6 and 9.8 mg/mL, respectively. Owing to ethanol as a solubilizing agent and a component of the ethosome formulation, the skin permeability is enhanced [10]. Thereby, in this study, 10-50% v/v ethanol was added in dispersion media of ethosome formulations. However, both the results show that no significant differences in the solubility of MPA in various pH and ethanol concentration between 48 and 72 h ($p > 0.05$) which demonstrated that the solubility of MPA reached equilibrium.

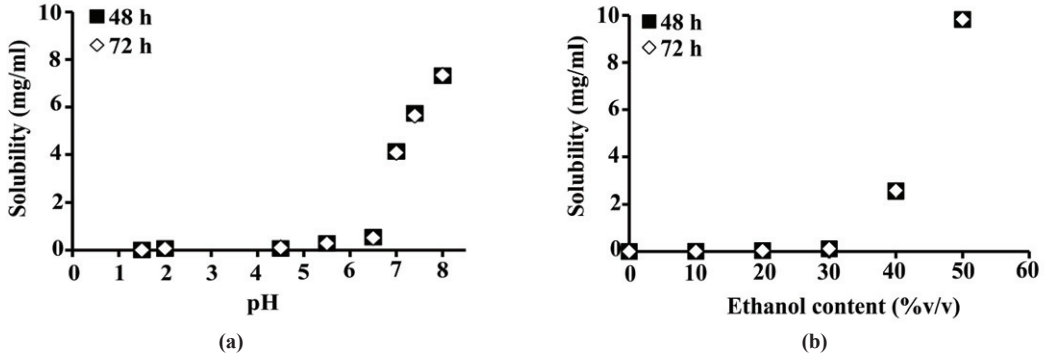

Fig. 2. (a) pH-solubility profile of MPA and (b) solubility profile of MPA in aqueous solutions with various ethanol concentrations. The plotted data are mean ± SD (n=5)

3.3. Formulation and preparation of ethosome containing MPA

In this study, the ethosome containing MPA was prepared and characterized to obtain the optimized formulation which may be suitable for application as a topical delivery system. The formulations were prepared according to the thin-film hydration method, using the concentration of MPA 10 mg/mL. The effect of ethanol and phospholipids at different concentration on the ethosome formulation were evaluated in terms of the physical appearance. It was found that the formulations containing 10-30% v/v ethanol and 2-6% w/v SPC (F.1-9) has a yellowish colloidal appearance which no precipitation of free drug, Figure 3. This may cause a synergistic effect between pH and ethanol as a co-solvent system with micelle solubilization of phospholipids, which can increases the solubility of MPA in the formulation. However, when increasing concentrations of ethanol up to 40 and 50% v/v in phosphate buffer pH 7.4 (F. 10-15), it was found that the MPA-loaded ethosome could not be performed due to precipitation of free drug as show in Figure 3. This can explain the phase separation of ethosome formulation as a result of the effect of high ethanol concentration on disruption of lipid vesicle, when further increase in the ethanol concentration probably made the vesicle membrane leak, thus leading to a decrease in entrapment efficiency which eventually leads to the precipitation of free drug. As 10-30% v/v ethanol in phosphate buffer pH 7.4 provided the best physical characteristics, this formulation was selected for further experiments.

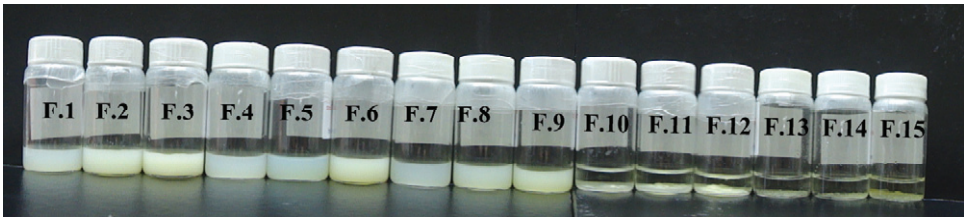

Fig. 3. Physical appearances of the ethosome prepared from 2, 4 and 6% w/v SPC using 10-50% v/v ethanol in phosphate buffer pH 7.4 as dispersion media; F.1,2,3 was 2, 4 and 6% w/v SPC using 10%v/v ethanol; F.4,5,6 was 2, 4 and 6% w/v SPC using 20% v/v ethanol; F.7,8,9 was 2, 4 and 6% w/v SPC using 30% v/v ethanol; F.10,11,12 was 2, 4 and 6% w/v SPC using 40% v/v ethanol; F. 13,14,15 was 2, 4 and 6% w/v SPC using 50% v/v ethanol, respectively

Table 1 shows the properties of the MPA ethosome. Formulation Etho-1, -5 and -9 without ethanol content as a liposome showed a high entrapment efficiency. However, the aggregation of lipid components was observed within one week. This may be due to the unsuitability of the formulation, which contained only SPC in the main lipid vesicle without other additives. The aggregation of lipid vesicle may occur due to electrostatic and hydrogen bonding interaction between choline head group of SPC**.** Since particle size and ethanol concentrations influence the permeability of the skin. The small particle size can penetrate through the skin over the larger particle size and the permeation of drug increased with the increase of ethanol concentration [10,11]. The main component of ethosome formulation will focus on the MPA ethosome that has the smallest particle size and appropriate ethanol. The concentration is important for increasing skin permeation of drug. Thereby, the formulation Etho-8 including 4% w/v SPC and 30% v/v ethanol with smallest particle size (154 ± 2 nm) was selected as the main component of MPA ethosome for further development. The main composition of MPA ethosome was developed to improve its entrapment efficiency by adding an addition such as CHOL, Tween80, PEG and surface charge agent, such as SA and DA and it was evaluated to select the optimized MPA ethosome as shown in Table 2.

Table1. The main compositions and physical characteristics of ethosome and liposome formulations

No.	Composition		Vesicle size (nm)	Polydispersity Index (nm)	Zeta potential (mV)	Entrapment efficiency (%)
	SPC(%w/v)	Ethanol(%v/v)				
Etho-1	2	0	415 ± 8	0.27 ± 0.02	-29 ± 3	58 ± 1
Etho-2	2	10	320 ± 8	0.32 ± 0.02	-48 ± 3	23 ± 0
Etho-3	2	20	332 ± 12	0.36 ± 0.01	-66 ± 8	23 ± 1
Etho-4	2	30	158 ± 3	0.29 ± 0.01	-63 ± 4	13 ± 0
Etho-5	4	0	440 ± 8	0.34 ± 0.01	-28 ± 6	78 ± 0
Etho-6	4	10	349 ± 6	0.35 ± 0.02	-67 ± 9	41 ± 0
Etho-7	4	20	622 ± 14	0.36 ± 0.06	-71 ± 13	24 ± 2
Etho-8	**4**	**30**	**154 ± 2**	**0.29 ± 0.01**	**-80 ± 7**	**17 ± 0**
Etho-9	6	0	453 ± 4	0.34 ± 0.02	-25 ± 7	82 ± 0
Etho-10	6	10	361 ± 14	0.33 ± 0.01	-75 ± 4	41 ± 1
Etho-11	6	20	464 ± 17	0.34 ± 0.02	-83 ± 5	25 ± 0
Etho-12	6	30	261 ± 4	0.30 ± 0.02	-82 ± 8	17 ± 1

Each data represents the mean±S.D. (n = 3).

Ethosome properties were improved probably due to the additional various additives of the formulation. CHOL is a stabilizing agent which prevents leakage and reduces the membrane permeability

of the vesicles leading to an increase in both size and efficient entrapment. Its molecule was inserted into lipid bilayer whereas the hydroxyl group was connected to the aqueous phase. The aliphatic chain paralleled to the acyl chains of SPC that result to the rigidity in the lipid vesicles increasing from intravesicle interaction. The addition of CHOL in formulations No. Etho-13, -14, -15 and -16 resulted in an increase of drug entrapment efficiency compared to formulation No. Etho-8. As well as, Tween80 that could enhance both the efficient entrapment and particle size (Etho-17) from its solubilizing effect. Moreover, the enhanced stability of the vesicles may be due to the hydrocarbon tail ability to penetrate into the lipid bilayer thus leaving the polyethylene oxide groups on the surface of the vesicle thereby introducing a steric barrier on the surface. It decreased in fusion of the vesicles and the exchange between lipid and drug when the vesicles particles collide. The effects of positive and negative charge agent, SA and DA, on ethosome characteristics were evaluated. The formulations with added SA greatly increased the particle size but the entrapment efficiency had decreased (Etho-18). However, the aggregation of lipid components was observed within one week. This may due to the incompatibility between the positive charge of SA and the negative charges of SPC and MPA. In contrast, the negative charge agent of DA could increase the entrapment efficiency of ethosome (Etho-16 and -13) when compared to formulations No. Etho-19 and -20, respectively, because of high interbilayer distance by electrostatic repulsive forces. The increase of negative surface charge of ethosome can also help to prevent vesicular aggregation. Besides, the effect of PEG on the ethosome formation in formulations No. Etho-26 and -27 showed in an increased of particle size compared to formulation No. Etho-16 and -13, respectively. The appropriate formulation, is dependent not only on the additives but also on other factors such as influence of ethanol and phospholipids, the concentration ratio, type of drug incorporated, as well as the method of preparation.

Table 2. The main compositions (4% w/v SPC, 30% v/v ethanol) with the adding of additives and physical characteristics of ethosome

No.	Additives composition	Ratio		Vesicle size (nm)	Polydispersity Index (nm)	Zeta potential (mV)	Entrapment efficiency (%)
		Molar	Weight				
Etho-8	-	-		154 ± 2	0.29 ± 0.01	-80 ± 7	17 ± 0
Etho-13	SPC:CHOL	3:1		162 ± 2	0.22 ± 0.01	-34 ± 3	19 ± 1
Etho-14	SPC:CHOL	4:1		474 ± 20	0.34 ± 0.02	-64 ± 2	26 ± 0
Etho-15	SPC:CHOL	3:2		707 ± 20	0.35 ± 0.02	-57 ± 4	62 ± 1
Etho-16	SPC:CHOL	2:1		258 ± 5	0.28 ± 0.04	-58 ± 4	19 ± 0
Etho-17	SPC:Tween80	4:1		190 ± 1	0.17 ± 0.03	-48 ± 2	30 ± 1
Etho-18	SPC:CHOL: SA	2:1:1		1899 ± 2	0.39 ± 0.04	31 ± 1	5 ± 2
Etho-19	SPC:CHOL:DA	2:1:1		546 ± 33	0.35 ± 0.03	-53 ± 7	34 ± 1
Etho-20	SPC:CHOL:DA	6:2:1		420 ± 2	0.32 ± 0.03	-53 ± 8	58 ± 2
Etho-21	SPC:CHOL:Tween80	2:1:1		190 ± 1	0.13 ± 0.01	-43 ± 3	21 ± 0
Etho-22	SPC:CHOL:Tween80	3:2:1		198 ± 2	0.09 ± 0.02	-28 ± 2	29 ± 1
Etho-23	SPC:CHOL:Tween80	6:2:1		173 ± 3	0.12 ± 0.05	-31 ± 5	12 ± 1
Etho-24	SPC:CHOL:Tween80:DA	2:1:1:1		131 ± 17	0.27 ± 0.03	-39 ± 4	35 ± 0
Etho-25	**SPC:CHOL:Tween80:DA**	**6:2:1:1**		**371 ± 8**	**0.27± 0.02**	**-46 ± 5**	**56 ± 1**
Etho-26	SPC:CHOL: PEG4000	2:1:1		525 ± 8	0.31 ± 0.07	-34 ± 3	9 ± 1
Etho-27	SPC:CHOL: PEG4000	6:2:1		305 ± 4	0.21 ± 0.06	-64 ± 2	32 ± 1
Etho-28	SPC : Tween80		84:16	259 ± 7	0.25 ± 0.03	-50 ± 2	33 ± 2
Etho-29	SPC : Tween80 : DA		84:16:25	462 ± 7	0.22 ± 0.05	-35 ± 4	16 ± 1

Each data represents the mean±S.D. (n = 3). SPC, L-α-phosphatidylcholine from soybean; CHOL, Cholesterol from lanolin; DA, deoxycholic acid; SA, stearylamine. Tween80, polyoxyethylene sorbitan monooleate.

The optimized MPA ethosome was selected from the particle size less than 500 nm, a narrow size distribution, Zeta potential more than 30 mV and entrapment efficiency more than 50%. The results of our studies were found that both the Etho-20 and -25 formulations were matched to the criteria set. However, the Etho-25 has a smaller particle size and narrower size distribution compared to the Etho-20. The effective entrapment of drug has similarity. Therefore, the Etho-25 including 4% w/v SPC:CHOL: Tween80:DA (6:2:1:1 molar ratio) as a lipid component and 30% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium was selected as the optimized MPA ethosome. This formulation gave ethosome with 371 ± 8 nm vesicular size (PI = 0.27 ± 0.02 nm), the zeta potential of -46 ± 5 mV and the efficient entrapment of 56 ± 1%.

4. Conclusion

In this contribution, we developed the ethosome containing MPA to apply for a topical delivery system. The suitable ethosomal formulation (Etho-25) was composed of 4% w/v SPC with CHOL, Tween80 and DA as additives. The molar ratio of SPC:CHOL:Tween80:DA was 6:2:1:1, the dispersion medium was 30% v/v ethanol in phosphate buffer pH 7.4. This formulation gave ethosome with 371 ± 8 nm vesicular size (PI = 0.27 ± 0.02 nm), the zeta potential of -46 ± 5 mV and the entrapment efficiency of 56 ± 1%. However, its stability and *in vitro* skin permeation study will have evaluated in the future.

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