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Original Research Article

Enhanced stability of phenylethyl resorcinol in elastic vesicular formulations

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Abstract

Purpose: *To enhance the stability and reduce photo-degradation of phenylethyl resorcinol (PR) by elastic vesicle formation.*
Methods: *PR solution was stored at different temperatures, pH conditions, and under protected and unprotected natural light. The color of the solution and total active content were investigated. Three types of elastic vesicles, viz, ethosomes, transfersomes and invasomes, were prepared and their sedimentation in formulations and total active content were investigated before and after storage under various conditions for 4 months. The stability of the solutions and vesicular formulations were assessed.*
Results: *PR solution was unstable at pH 9, higher temperature (70 ± 1 ^{o}C) and under natural light. The color of PR solution changed from colorless to orange tone and the PR content decreased. On the other hand, PR entrapped within ethosome, tranfersome and invasome vesicles showed better stability, color change was not observed in the formulations, and PR content remained > 90 %.*
Conclusion: *All the vesicles display reduced degradation of PR under thermal and natural light. Thus, PR vesicular formulation enhances stability and improves the quality of the product for use in topical administration.*

Keywords: *Phenylethyl resorcinol, Degradation, Ethosomes, Transfersomes, Invasomes, Topical administration*

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INTRODUCTION

Phenylethyl Resorcinol (4 - (1- Phenylethyl) 1, 3-Benzenediol, PR) is a recently introduced skin-lightening agent that can interrupt tyrosinase activity by obstructing tyrosine being converted to L-3,4-dihydroxyphenylalanine (L-DOPA) [1]. The previous research reported that PR has more effective tyrosinase inhibition compared to kojic acid, both *in vitro* epidermal model (MelanoDerma™) and *in vitro* mushroom tyrosinase, approximately 22 times more [2]. In addition, it served as an antioxidant, which is more effective than butylated hydroxytoluene (BHT), ascorbic acid (vitamin C), and alpha tocopherol (vitamin E) [3]. Moreover, recently it has also shown a significantly higher antifungal activity than the antifungal agent fluconazole [4]. Nevertheless, the formulation of PR as topical products is limited because of the poor water

solubility and instability in light which leads to the decrease in effectiveness when used [5].

Several researches reported that the stability and solubility of the active ingredient improved by nano-encapsulation techniques. According to the previous researches, the vesicle carriers were studied; for example, liposome and nanostructure lipid carrier (NLC). The PR showed stability for at least 90 days when encapsulated within liposome [6]. In addition, after loading the PR into NLC, it was found that there was excellent physiochemical stability under day light over 3 months [7]. These researches supported that the photo-stability and solubility of the vesicle carriers can be increased by protection of encapsulated actives from the external environment. It was reported that the elastic vesicles not only improved solubility and photo-stability, but also improved permeation of active ingredients through/to the skin [8-11]. Therefore, in this study, elastic vesicle carriers such as PR-loaded ethosomes, transfersomes and invasomes were assessed for overcoming the limitation of using PR for topical products.

EXPERIMENTAL

Materials

PR was obtained from Starchem Enterprises Limited (Nanjing, China). L-α-phosphatidylcholine from soybean (SPC) and cholesterol (CHOL) were obtained from Sigma-Aldrich (Missouri, USA). Absolute ethanol was obtained from RCI Labscan Limited (Bangkok, Thailand). (R)-(+)-Limonene and (1R)-(−)-Fenchone were obtained from Sigma-Aldrich (WGK, Germany). Sodium deoxycholate (SDC) were obtained from Homedia® (Mumbai, India). Milli-Q water was prepared by Faculty of Pharmaceutical Sciences, PSU. None of the chemicals obtained were modified, and they were all used in the same state as they were received.

Solubility study of PR

Since the solubility property influences the preparation of vesicle formulation, the effects that ethanol and pH concentrations can have on PR solubility were evaluated. The effect of ethanol concentrations on PR solubility was studied to discover the suitable dispersion medium for preparing the ethosomes containing PR. The solubility of PR in ethanol : water mixed at various ethanol concentrations was determined. This study was carried out by adding an additional amount of PR into glass vials containing 5 ml of various ethanol concentrations (0, 10, 20, 30, 40, 50, 80, and 100 % v/v in water). These samples were then sonicated for 30 minutes and preserved at room temperature (30 ± 1 ºC). After 48 and 72 hours, samples were tested to determine if the solubility of PR has reached equilibrium. Solutions which were saturated were filtered by a 0.45 µm syringe filter membrane, then diluted with methanol, and finally analyzed for PR content at 254 nm using high-performance liquid chromatography (HPLC). These experiments were reciprocated for 3 times, and all the samples were examined in triplicate.

The effect of pH conditions on PR solubility was evaluated to find out the suitable receptor medium system in skin permeation study. The effect of pH on PR solubility was studied at four different pHs (2.0, 5.5, 7.4 and 9.0). An excess amount of PR was added in glass vials holding 5 ml hydrochloric acid buffer having pH 2.0, acetate buffer having pH 5.5, and phosphate buffer having pH 7.4 and 9.0 which was prepared in consonance with USP 30 [12]. These samples were first sonicated for 30 minutes, then preserved at room temperature (30 ± 1 ºC) for 48 and 72 hours to determine if the solubility of PR has reached equilibrium. Solutions which were saturated were filtered by a 0.45 µm syringe filter membrane. Afterwards methanol was used to dilute them, before determining the PR content using HPLC at 254 nm. These experiments were reciprocated for 3 times, and all the samples were examined in triplicate.

Degradation study of PR

The stability and degradability of PR under various pH, temperature, and light conditions were studied following the method explained by Ding *et al* [13]. The thermal stability of PR was studied at 4, 30, 45 and 70 °C under controlled humidity at 75 % RH. 50 mg of PR was dissolved in 250 ml of phosphate buffer having pH 7.0 to prepare the sample solution with a final concentration of 200 µg/ml. A 20 ml of sample solution was displaced into test tubes, capped to avoid evaporation and covered with aluminum foil to protect from light. Test tubes were kept in a refrigerator at 4 ± 1 ^{o}C, stored at 30 ± 1 ^{o}C, 45 ± 1 ^{o}C and 70 ± 1 ^{o}C inside a Constant Climate Chamber (Model HPP260, Memmert, Schwabach, Germany), respectively. At the temperature 4, 30 and 45 °C, 500 µl of supernatant was withdrawn to determine the content of PR at 0, 0.5, 1, 5, 9, 12, 24 and 30 days. At the temperature of 70 °C, the content of PR was studied at 0, 3, 6, 9, 12, 15, 24 and 30 hours. The supernatant was filtered using a 0.45 µm syringe filter membrane and was examined

using HPLC at 254 nm. These experiments were reciprocated for 3 times, and all the samples were examined in triplicate.

The effect of pH on PR stability was studied at four different pHs (2.0, 5.5, 7.4 and 9.0) at the room temperature (30 ± 1 °C). An accurate concentration of PR (200 µg/ml) in different pHs was prepared. Each buffer solution was transferred equally into three test tubes. Then, it was capped tightly to avoid evaporation and covered by aluminum foil to protect from light. 500 µl of sample was removed at 0, 0.5, 1, 3, 5, 7, 14, and 30 days to determine the content of PR using HPLC. These experiments were reciprocated for 3 times, and all the samples were examined in triplicate.

The effect of light on PR was studied to evaluate the photo-stability. 200 µg/ml of PR in phosphate buffer pH 7.0 was prepared and a 20 ml sample was transferred into test tube. After that, it was capped tightly to avoid evaporation and stored in room temperature under light protection by covering with aluminum foil and under natural light. On study day 0, 0.5, 1, 3, 5, 7, 14 and 30 days, the 500 µl of sample was collected, filtered using a 0.45 µm syringe filter membrane and analyzed using HPLC to determine the concentration of PR. These experiments were reciprocated for 3 times, and all the samples were examined in triplicate.

Kinetic analysis

The concentration of PR remaining was calculated and expressed in mean ± standard error. Degradation rate kinetics was determined by plotting concentration versus time (zero-order reaction), logarithm of concentration versus time (first-order reaction) and concentration^{-1} versus time (second-order reaction). The correlation coefficients (r^2) were calculated and the best fit for indicating the reaction order was determined. The kinetics models can be represented as in Eqs 1 - 3 [14]:

Zero-order reaction:

$C = C_0 - kt$

$t_{1/2} = C_0/2k$ ……. (1)

First-order reaction:

$\ln C = \ln C_0 - kt$

$t_{1/2} = \ln 2/k$ …….. (2)

Second-order reaction:

$1/C = 1/C_0 + kt$

$t_{1/2} = 1/kC_0$ …….. (3)

where C_0 = the concentration of the reactants under consideration at time zero, C the concentration after reaction time (t) and k the reaction rate constant.

Preparation of vesicular formulations

In this study, the ethosomes, transfersomes and invasomes were selected to study on improving stability of PR. All vesicles were prepared accounting to the method of our previous reports [15-17]. The main composition of all vesicles composed of 3 % w/v SPC: 0.5 % w/v CHOL: 0.5 % w/v PR: up to 100 % v/v water phase. The penetration enhancer absolute ethanol, SDC and D-limonene mixed with 10 % v/v absolute ethanol were added into the main composition for ethosomes, transfersomes and invasomes, respectively. Thin film hydration method was used to prepare these compositions. Finally, the physical property and total active content were evaluated at initial time and after storing at 4 ± 1, 30 ± 1 and 45 ± 1 °C at 75 % RH in a Constant Climate Chamber (Model HPP260, Memmert, Schwabach, Germany) for 4 months. In addition, all formulations were prepared for the morphology study according to the method explained by Limsuwan *et al* and Amnuaikit *et al* [16,17]. The surface morphology and the structure within the vesicles were observed by scanning electron microscopy (SEM, Quanta 400, FEI, Czech Republic) and transmission electron microscopy (TEM, JEM-2010, JEOL, Japan), respectively.

Quantitative determination of PR

The quantitative determination of PR in all experiments was carried out using HPLC method which was followed by Limsuwan *et al* [18]. Analysis was done using Agilent 1100 series with a BDS HYPERSIL C18 column (150 x 4.6 mm, 5 µm) at 25°C was used for analysis. The mixture of acetonitrile, methanol, milli-Q water as 40:20:40% v/v/v was used as mobile phase, maintained at 0.8 ml/min. Then, sample solution of 20 µl was injected, and quantity of the PR was detected at 254 nm.

Statistical analysis

The data collected from this study were presented as mean ± standard deviation (SD). Student t-test or one-way analysis of variance (ANOVA) followed by post hoc analysis was used to test the statistical significant of

differences between or among groups. The statistically significant value was considered at P < 0.05.

RESULTS

Ethanol-solubility profile

The effect of ethanol concentrations on PR solubility were studied, the results were expressed as ethanol-solubility profile in Figure 1 A. PR exhibited that it was slightly soluble in water (318.83 ± 22.02 µg/ml at 25 °C for 72 h) which is similar to the previous report of PR solubility (265 µg/ml at 25 °C) [15].

pH-solubility profile

pH-solubility profile *(*Figure 1 B) showed that the solubility of PR in acidic range was higher than alkaline range. The solubility PR in pH 1.5 and pH 2.0 were higher than the solubility PR in pH 7.4 and pH 8.0. These results indicated that the PR solubility decreased when the pH increased.

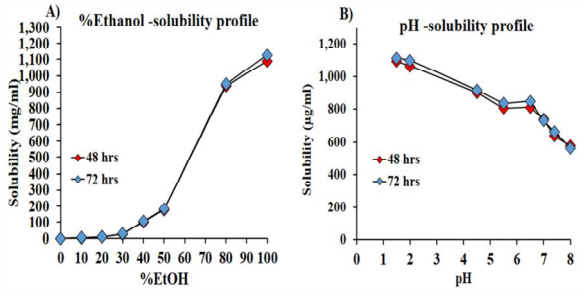

Figure 1: The ethanol (A) and pH -solubility profile (B) of PR at room temperature (30 ± 1 ºC) for 48 and 72 hours. Each value represents the mean ± SD (*n* = 3)

Effect of temperature on PR

Figure 2 A - D shows PR concentration after storing at 4 ± 1, 30 ± 1, 45 ± 1 °C and 70 ± 1 °C under controlled humidity at 75 %RH. After the samples were kept at 4 ± 1, 30 ± 1, 45 ± 1 °C for 30 days, the physical property and PR concentration remained unchanged. Whereas, after storing the sample at 70 ± 1 °C for 30 hours, the PR concentration decreased from 183.96 ± 0.38 to 164.78 ± 5.5 µg/ml.

Effect of pH on PR

The effect of four different pH values (2.0, 5.5, 7.4 and 9.0) on PR stability studied at the room temperature (30 ± 1 °C) are shown in Figure 2 E-H. The PR concentration in pH 2.0, 5.5, 7.4 demonstrated that it slightly decreased after storing for 30 days; on the other hand, the PR concentration in pH 9.0 highly decreased. At pH 9.0, the PR concentration decreased from 93.48 ± 1.16 to 73.48 ± 3.61 %. Therefore, PR is stable in pH ranging from 2-7.4, whereas it is not stable at pH 9.

Effect of natural light on PR

Figure 2 shows the PR concentration after storing under natural light (Figure 2 I) and under light protection (Figure 2 J). It was stable under light protection because there was no change in the PR concentration and the color of PR solution (Figure 3, LP1 - LP3). In contrary, when the PR solution was kept under natural light, the PR content was decreased. The PR concentration at initial time was 93.13 ± 0.31 % while the PR concentration under natural light at 30 days was 54.80 ± 8.75 % (Figure 2 I). In addition, the color of PR solution after storing for 30 days changed to orange color as shown in Figure 3, NL1 - NL3. Our results clarified that the PR solution was not stable under light condition. This is a problem when it comes to formulation of PR cosmetic and skin dermal products. Therefore, overcoming the limitation of using PR as topical product by increasing the light stability is necessary. In this study of nanoencapsulation techniques; ethosomes, transfersomes and invasomes were selected to increase light stability of PR. These systems were not only protecting the encapsulated actives from external environment, but also improving the skin permeation of PR through/to the skin.

The kinetic study

After collecting the data, the linear regression was plotted between time point's and stability test results following each kinetic model.

The slope of the regression equation was evaluated to confirm the goodness of fit of the linear regression. If the *p*-value of slope is larger than or equals to 0.05 that means the slope of regression equation equals to zero. It represents that there is no change in stability test results under the studied conditions. In addition, r^2 value was calculated which was near to 1, it represents the best fit with the reaction order. Table 1 shows the r^2 and *p*-value of kinetic parameters of PR degradation in storage conditions, like temperature, pH and light.

The stability results showed that the stability of PR remained constant under conditions of 4 ± 1, 30 ± 1 and 45 ± 1 °C, pH 5.5 and light protection (*p*-value > 0.05). In contrast, the regression equation of storage of PR under 70 ± 1 °C, pH 2, pH 7.4, pH 9 and natural light gave the linear

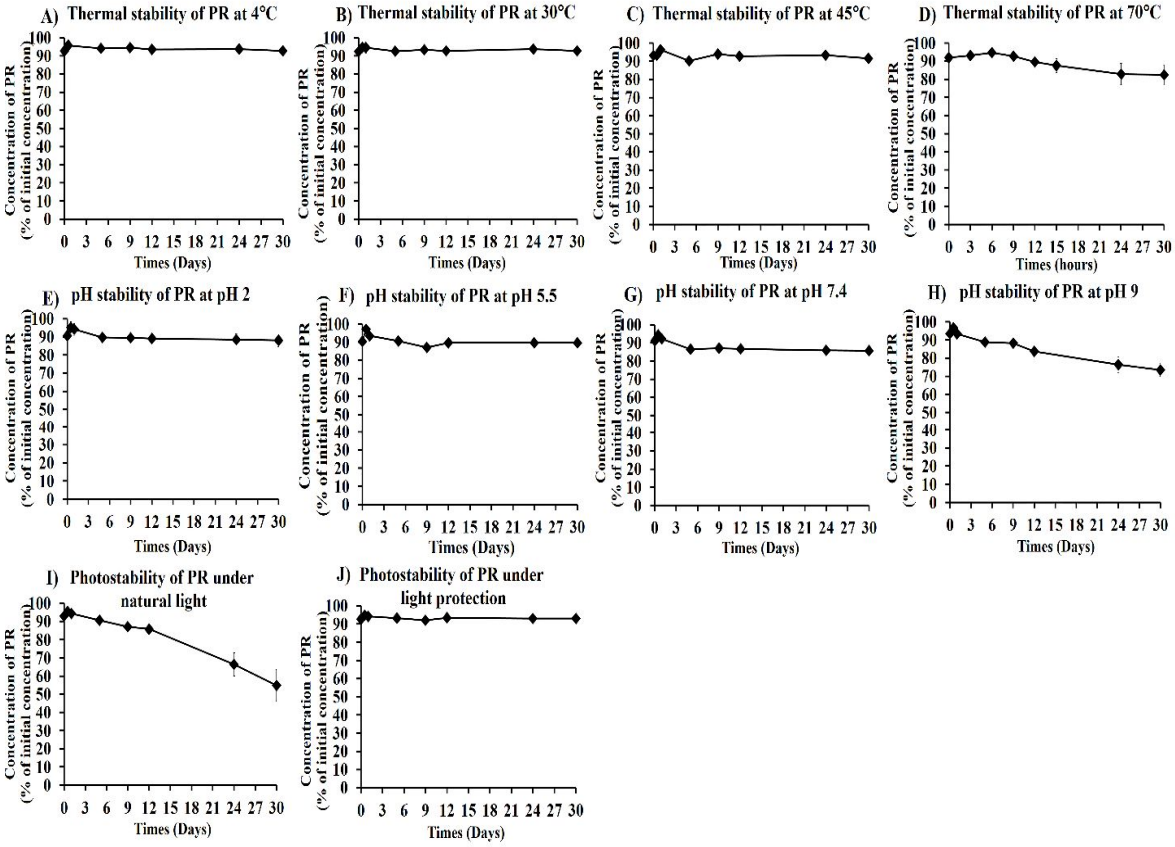

Figure 2: The effect of storage conditions on the PR concentration (200 µg/ml), thermal stability (A – D), pH stability (E – H), and photostability (I – J). Each value represents the mean ± SD (*n* = 3)

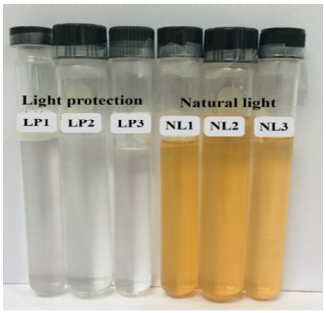

Figure 3: Color of PR solution after stored under light protection (LP1 - LP3) and under natural light (NL1-NL2) for 30 days

regression (p-value < 0.05). It indicated that there was degradation of PR under these conditions.

The degradation of PR after storing at 70 ± 1 °C and pH 9 were fitted to second-order reaction model. The highest r^2 values were 0.8758 and 0.9816 which were obtained from second-order reaction model under condition at 70 ± 1 °C and pH 9, respectively. In case of light conditions, the degradation of PR when it was unprotected under light indicated the zero-order reaction model (r^2 = 0.9671). Therefore, under natural light, PR in solution form degraded when compared to the initial concentrations. However, the effect of pH 2 and pH 7.4 on PR degradation was not clear in the times of study.

Effect of temperature on vesicular formulations containing PR

The effect of different temperatures on the color change and total active content of PR formulations were evaluated. Figure 4 and Figure 5 showed that the effect of storage conditions on color change and total active content of the ethosomes, transfersomes and invasomes at 4 ± 1, 30 ± 1 and 45 ± 1 °C / 75 % RH for 4 months, respectively. The color of PR-ehtosome and transfersome formulations remained unchanged after storing at 4 ± 1 and 30 ± 1 °C for 4 months (Figure 4), whereas, color change of PR-invasomes was observed after storing for 1 month, and followed by the precipitation of the formulations (Figure 4). This represents the instability of PR-invasomes at 4 ± 1 °C. However, after increasing temperature of storage to 45±1°C, the instability was found in all formulations in 4 months. The color changed to orange, and the precipitation of active ingredient or other formulation were observed (Figure 4). In case of the total active content, all formulations

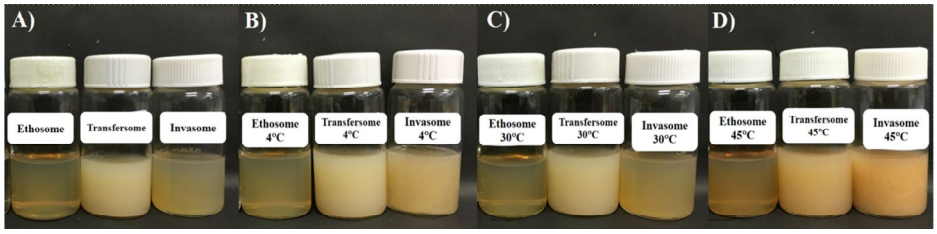

Figure 4: Sedimentation of PR-ethosomes, transfersomes and invasomes before (A) and after storage at 4 ± 1 °C, (B), 30 ± 1 °C / 75 % RH (C) and 45 ± 1 °C / 75 % RH (D) for 4 months, respectively

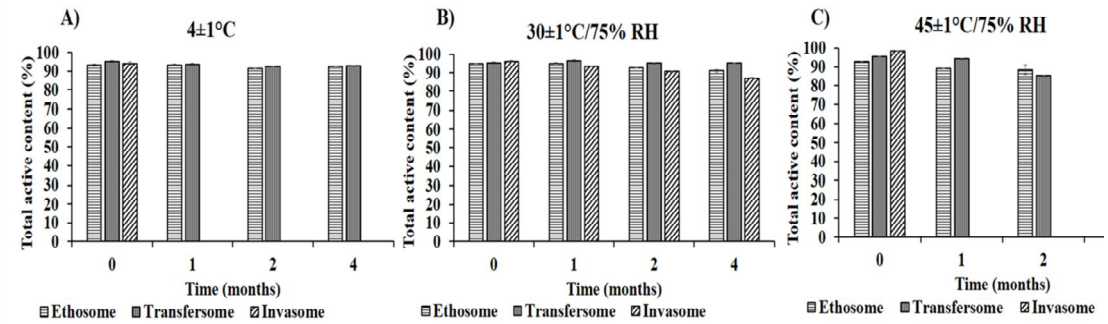

Figure 5: Effect of storage conditions on total active content of the ethosomes, transfersomes and invasomes at 4 ± 1 °C, 30 ± 1 °C / 75 % RH and 45 ± 1 °C / 75 % RH for 4 months, respectively

showed that the total active content was approximately 90 % after storing at 30 ± 1 °C. However, after they were stored at 4 ± 1 °C and 45 ± 1 °C, there were only two systems which were stable, including ethosomes and transfersomes. They had a total active content of more than 90 % over 4 months at 4 ± 1 °C, and more than 85 % for 2 months at 45 ± 1 °C. All the results showed that the optimized temperature for storage of these systems was 30 ± 1 °C.

Effect of natural light on vesicular formulation

In this study, we focus on the effect of natural light on the change of color and total active content of PR. The PR was entrapped within several vesicular formulations, like ethosomes, transfersomes and invasomes. Since the previous study found that after storing the PR solution under natural light at room temperature for 30 days, its color changed from colorless to orange color (Figure 3). In addition, the PR content also decreased about 38.33 % from the initial PR (Figure 2 I). These results indicated the instability of PR in natural light.

On the other hand, when the PR was entrapped within the ethosome, tranfersome and invasome formulations, it showed that the color of all formulations remained unchanged over 4 months after storage under natural light as shown in Figure 4 C. Moreover, the total PR content in the formulation also decreased slightly (Figure 5).

Table 1: Kinetic parameters of PR degradation

Storage conditions		Kinetic parameter of degradation					
		Zero-order		First-order		Second-order	
		P-value	r^2	*P-value*	r^2	*P-value*	r^2
Thermal stability	4 ± 1 °C	0.1588	-	0.1593	-	0.1598	-
	30 ± 1 °C	0.5654	-	0.5565	-	0.5596	-
	45 ± 1 °C	0.4222	-	0.4272	-	0.4324	-
	70 ± 1 °C	0.0008	0.8661	0.0007	0.8713	0.0006	0.8758
pH stability	pH 2	0.0404	0.5307	0.0381	0.5389	0.0359	0.5471
	pH 5.5	0.2179	-	0.2194	-	0.2212	-
	pH 7.4	0.0308	0.5679	0.0293	0.5747	0.0278	0.5814
	pH 9	0.0000	0.9655	0.0000	0.9749	0.0000	0.9816
Photo stability	Under light protection	0.3837	-	0.3862	-	0.3889	-
	Under natural light	0.0000	0.9671	0.0000	0.9463	0.0002	0.9190

Note: Data are mean ± SEM (n = 5)

shows the effect of storage conditions on total active content of the ethosomes, transfersomes and invasomes at 4 ± 1 °C, 30 ± 1 °C / 75 % RH and 45 ± 1 °C / 75 % RH for 4 months, respectively. These results shows that decrement of total PR content at 30 ± 1 °C / 75% RH in case of ethosomes, tranfersomes and invasomes were 4.04, 9.12 and 2.50 % of the initial PR, respectively. Figure 6 shows the surface morphology of PR ethosome, transfersome and invasome vesicles when observed by SEM and TEM. SEM photographs indicated spherical structure in all vesicular systems (Figure 6 A - C) while TEM photographs demonstrated unilamellar vesicle structure for invasomes (Figure 6 F), and uni- to multi-lamallar vesicle structure for ethosomes (Figure 6 D) and transfersomes (Figure 6 E).

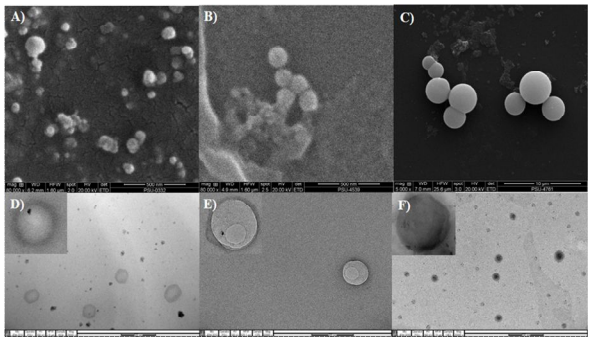

Figure 6: SEM photographs of ethosome (A), transfersome (B) and invasome formulations (C), and TEM photographs of ethosome (D), transfersome (E) and invasome formulations (F), respectively

DISCUSSION

The solubility property influences formulation and preparation of vesicular formulation. Since PR is hardly soluble in water at all (265 µg/ml at 25 °C), the solubility of PR in various pH values and ethanol concentrations were studied to discover a suitable dispersion medium for the vesicular formulations. There were no significant differences in the solubility of PR in various pH condition and ethanol concentrations between 48 and 72 h, displayed no noticeable differences, which indicated that the solubility of PR had reached equilibrium. The low water solubility of PR may be due to the inability of water, to break into the lattice structure of PR [19]. On the other hand, when the co-solvent systems were used, the PR solubility increased when the ethanol concentration increased. For example, the 10 % ethanol gave the PR solubility of 5.01 ± 0.04 mg/ml whereas the 50 % ethanol gave the increased PR solubility of 181.44 ± 1.29 mg/ml at 25 °C for 72 h. The solubility enhancement of PR is probably due to the H-bonding interactions of ethanol, because it has both hydrogen bond donor and acceptor properties.

The low stability of PR at very high temperature may be due to the increase in rate of a reaction with increase in temperature. The active ingredient is responsive to degradation at higher temperature through various reactions like hydrolysis, pyrolysis, polymerization, isomerization, rearrangement, and decarboxylation [20]. In fact, light can act as a catalyst to oxidation reactions. Since, PR has low stability in natural light when it is exposed to the light, the color of PR solution changes from colorless to orange or pink tone, which may reduce the effectiveness of PR [5].

The instability of the vesicles may be because of the higher fluidity of lipid bilayers after storing at high temperature. It resulted in higher drug leakage and finally leaded to precipitation of the formulations [15]. In the case of the slight decrease in PR total active content of vesicular formulations, it may be the result of the encapsulation of PR within the vesicles. Therefore, these vesicles could reduce the degradation of PR under natural light, leading to improvement of the physical property, the content of the PR remaining, and the duration of formulation stability. The improvement of PR stability by these vesicles as a result of the encapsulation of PR within the vesicles, which protects the PR from external environment leads to the decrease in degradation reactions, for example hydrolysis and oxidation reactions. In case of transfersomes, the PR was encapsulated at lipid bilayer of the vesicles. In contrast, ethosomes and invasomes which composed of ethanol in the formulation, the PR could be distributed throughout the vesicle [15-17]. The encapsulation of PR within the vesicles helps to protect the PR from the degradation by natural light, resulting in the higher stability for long periods of time.

CONCLUSION

Formation of vesicles reduces the degradation of PR under thermal and natural light conditions, resulting in the color remaining constant, reduced degradation of PR content, and higher stability.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contributions of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The experiments in this study were done by Tunyaluk Limsuwan. Prapaporn Boonme reviewed this manuscript and gave comments for designing this study. Thanaporn Amnuaikit drafted this manuscript and supervised the other authors.

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