AAPS PharmSciTech, Vol. 13, No. 1, March 2012 (© 2012)
DOI: 10.1208/s12249-012-9751-1

Research Article

Transdermal Absorption Enhancement of Rice Bran Bioactive Compounds Entrapped in Niosomes

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Received 18 August 2011; accepted 4 January 2012; published online 19 January 2012

Abstract. Niosomes composed of Tween 61 and cholesterol at 1:1 molar ratio were entrapped with the mixture of the three semi-purified rice (*Oryza sativa* L., Family Gramineae) bran bioactive compounds [ferulic acid (F), γ-oryzanol (O), and phytic acid (P)] at 0.5%, 1.5%, and 1.5%, respectively, by the supercritical CO_2 technique. The transdermal absorption by vertical Franz diffusion cells of the compounds entrapped in niosomes (Nio FOP), the unentrapped compounds (Mixed FOP), the compounds incorporated in gel and cream (Gel FOP and Cream FOP), and the compounds entrapped in niosomes and incorporated in gel and cream (Gel nio and Cream nio) was investigated. At 6 h, F and P from Nio FOP gave lower cumulative amount in viable epidermis and dermis (VED) than from Mixed FOP of 1.1 and 1.6 times, respectively, while O from Nio FOP exhibited higher cumulative amount in VED than from Mixed FOP of 2.4 times. The highest cumulative amount in VED of F, O, and P were from Gel nio, Cream nio, and Mixed FOP at 1.564±0.052, 15.972±0.273, and 25.857±0.025 ng/cm^2, respectively. Niosomes enhanced the transdermal absorption of the hydrophobic compound O, while retarded the hydrophilic compounds F and P indicating the less systemic risk of F and P than O when entrapped in niosomes. Thus, transdermal absorption of F, O, and P appeared to depend on niosomal size, lipophilicity of the bioactive compounds, and types of formulations. These preclinical results can be applied for the design of the clinical study of the developed rice bran niosomal topical products.

KEY WORDS: ferulic acid; gamma oryzanol; niosomes; phytic acid; rice bran extract; transdermal absorption.

INTRODUCTION

UV and other exogenous environmental factors can lead to the production of reactive oxygen species which is one of the causes of skin damage and aging (1,2). Topical application of antioxidants has been suggested as a preventive therapy for skin photo-aging and UV-induced skin cancer (3,4). Many natural antioxidants have been used in skin anti-aging products including bioactive compounds from rice bran such as ferulic acid (F), γ-oryzanol (O), and phytic acid (P) (5). F with the molecular weight of 194.18 g mol^{-1} is a hydrophilic ubiquitous polyphenol formed from the two amino acids (phenylalanine and tyrosine). O with the molecular weight of 602.9 g mol^{-1} is the ferulate esters of triterpene alcohols and phytosterols. P with the molecular weight of 660.04 g mol^{-1} is a natural hydrophilic component found in plant fiber and also known as phytate, inositol hexaphosphate, and IP-6. These compounds have been reported to have cosmetic effects including UV filters and free radical scavengers. However, their antioxidative properties are usually destroyed when expose to air and light (6). Moreover, their improper lipid to water partition coefficient (log *P* value), solubility, and large molecular size are the limitations of skin permeation (7). Usually, for better penetration across the stratum corneum (SC) which is the major permeability barrier and the rate-limiting factor due to its highly organized lipid structures (8), the topical applied substances should have the optimum log *P* values of 1 to 3 (9) and the molecular weight of less than 500 g mol^{-1} (10,11). The less than 1 (12) of log *P* value of F indicates the too low lipid solubility which may limit the permeability through SC. For O, although it has the optimum log *P* value of around 3 (13), its high molecular weight may hinder the skin permeability. P is also difficult to penetrate across SC because of its high hydrophilicity (log *P* value<1) (14) and high molecular weight (15). One of several approaches which have been developed to enhance the penetration of many substances across SC is the application of niosomes. Niosomes were first reported in the 1970s as a carrier for cosmetics (16,17). Niosomes are closed bilayer structures

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formed from self-assembly of non-ionic surfactants such as Tween 61 or Span 60 in an aqueous media. Similar to liposomes, niosomes are capable to entrap both hydrophilic and hydrophobic substances and can serve as transdermal carriers. However, niosomes are more stable and cost-effective than liposomes (18,19). The objective of this study was to investigate the transdermal absorption of the three rice bran bioactive compounds [ferulic acid (F), γ-oryzanol (O), and phytic acid (P)] when entrapped in niosomes.

MATERIALS AND METHODS

Materials

Rice bran (Payom fresh market in Chiang Mai, Thailand) and wastes from the rice bran oil production which were acid-distilled by-products and crude rice bran oil (Kamolkij Group Company and Thai Edible Oil Co., Ltd., Thailand) were used to prepare the semi-purified rice bran extracts containing F, O, and P. Stearic acid (Srichand United Dispensary Co., Ltd., Thailand), mineral oil (O.V. Chemical & Supply, Thailand), cetyl alcohol (Vidhyasom Co., Ltd., Thailand), white petrolatum (Vechavit, Thailand), isopropyl myristate (Vidhyasom Co., Ltd.), glycerin (O.V. Chemical & Supply, Thailand), triethanolamine (Asia Pacific Specialty Chemicals Limited, Thailand), methyl paraben and propyl paraben (Vidhyasom Co., Ltd.), vitamin E acetate (Namsiang Co., Ltd., Thailand), propylene glycol (O.V. Chemical & Supply), and Carbopol 980 (Namsiang Co., Ltd.) were cosmetic grade. Tween 61 (polyoxyethylene sorbitan monostearate) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Cholesterol was purchased from Serva Feinbiochemica, Heidelberg, Germany. All other chemicals were of analytical reagent grade. The standards F, O, and P were from Fluka, Tsuno Rice Fine Chemicals Co., Ltd. in Japan, and Sigma-Aldrich Co. in Germany, respectively.

Preparation of Rice Bran Semi-purified Extracts Containing F, O, and P

The semi-purified rice bran extracts containing F, O, and P were prepared from the acid-distilled rice bran oil, crude rice bran oil, and the rice bran, respectively, by the method previously described (6). Briefly, the semi-purified extract containing F was prepared from the acid-distilled rice bran oil by dissolving in 95% ethanol (1:3 *w*/*v*) and then hydrolyzed with 0.5 M of NaOH in dark for 24 h and partitioned with diethyl ether. The semi-purified extract containing O was prepared from the crude rice bran oil and dissolved in ethyl acetate (1:3 *w*/*v*) and then semi-purified by silica gel-column chromatography with hexane/ethyl acetate (7:3) as an eluent. The F and O contents were determined by HPLC (HPLC, AS 1000, Thermo Finigan, USA) at 320 nm. The mobile phases for F and O were 80% methanol and methanol/dichloromethane/acetonitrile/acetic acid (55:35:9.5:0.5), respectively. The semi-purified extract containing P was prepared from the rice bran by dissolving in 2.4% hydrochloric acid (1:5 *w*/*v*) and then purified by Amberite IRA-400 (anion-exchange) resin–column chromatography with NaCl solution as an eluent. The content of P was determined by spectrophotometry (T80 UV/VIS spectrophotometer, PG instruments Ltd., China) at the wavelength of 690 nm (6).

Preparation of Niosomes by Supercritical CO_2 Fluid ($scCO_2$)

The niosomes were prepared by $scCO_2$ technique as previously described (20). Briefly, the total amount of 20 mM of Tween 61 mixed with cholesterol together with the semi-purified extracts containing F, O, and P at 0.5%, 1.5%, and 1.5% *w*/*w*, respectively, in distilled water (100 ml) were added into the stirred-view cell. The volume of the view cell was ca. 150 cm^3. The temperature in the view cell was raised to 60°C and the CO_2 gas was introduced into the view cell. The pressure and temperature in the view cell were maintained at 200 bar and 60±1°C, respectively. After 30 min with stirring until equilibrium, the pressure was released and the niosomal dispersion was obtained.

Characteristics of Niosomes Entrapped with the Semi-purified Extracts

Vesicular Size Determination

The vesicular size of niosomes was measured by dynamic light scattering apparatus (NICOMP 380 ZLS, Particle Sizing Systems, Santa Barbara, CA). The niosomal dispersion was diluted to about 100 times with distilled water. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90° and the wavelength at 535 nm.

Morphology

The dispersion of niosomes was rapidly frozen in liquid propane using cryo-preparation apparatus (Leica EM CPC, Leica Co., Vienna, Austria). The frozen sample was fractured in freeze-replica-making apparatus (FR-7000A, Hitashi Science Co., Tokyo, Japan) at −150°C. The fracture surface was replicated by evaporating platinum at an angle of 45°C and followed by carbon to strengthen the replica. It was placed on a 150 mesh copper grid after washing with acetone and water. The vesicles were observed under a transmission electron microscope (JEM-1200EX, JEOL Co.) with magnification of ×12.0k.

Transition Temperature Analysis

Thermal analysis of the niosomal dispersion was determined by a differential scanning calorimeter (DSC 8230, Rigaku Co., Tokyo, Japan). Twenty microliters of the niosomal dispersion were placed and sealed in the aluminum pan sample vessel. The measurement conditions were at 1 K/min for scanning rate, 20–90°C for the scanning range and 0.1 mcal/s for the sensitivity. Distilled water instead of an empty pan was used as a reference. If water is in the sample, it can act as a plasticizer and reduce transition temperatures. Also, water in the sample can be volatile during the run, causing an endothermic peak and a shift in the baseline. Since our samples contained water, water was used as a reference in this study to set the baseline (21,22). All measurements were determined in triplicate.

Niosomal Membrane Rigidity Analysis (5)

1, 6 diphenyl-1, 3, 5-hexatriene (DPH) in tetrahydrofuran (THF) was used as a fluorescent probe for the determination

of membrane rigidity by a fluorometer. The molar ratio of niosomes to DPH was 300:1. The mixture was incubated for 24 h at 45±1°C. The rigidity of niosomal membrane was determined by fluorescence polarization (*P*) which was calculated according to the following equation:

$$P = \frac{(I\mathrm{p} - GI\mathrm{v})}{(I\mathrm{p} + GI\mathrm{v})}$$

where *I*p and *I*v were the fluorescence intensity of the emitted light polarized parallel and vertical to the exciting light, respectively, and *G* was the grating correction factor. The fluorescence intensities (*I*p and *I*v) were measured at various temperatures by a spectrofluorophotometer. The excitation and emission wavelength were 350 and 450 nm, respectively. The measurements were performed in triplicate.

Entrapment Efficiency Determination

The entrapment efficiencies of F, O, and P containing in the semi-purified extracts entrapped in niosomes were determined by gel-filtration using Sephadex® G-50 (Amersham Biosciences Limited, Sigma-Aldrich) as a packing material and purified water as an eluent. Eluates were collected in tubes using a fractional collector (Foxy JR, Isco Inc., Lincoln, USA) at the flow rate of 7 ml/min. The fractions containing niosomes entrapped with the extracts detected at 470 nm (23) were pooled, collected, and dried with a freeze-dryer (CHIST, Martin Chist, Germany) at −52°C and 0.05 mbar for 24 h. The lyophilized powder was dissolved in absolute ethanol and assayed for F and O contents by HPLC. For the analysis of the P contents, the powder was dissolved in distilled water and assayed by colorimetry using a spectrophotometer at 690 nm. The percentages of entrapment efficiency were calculated according to the following equation:

$$\text{Entrapment efficiency}\,(\%) = \frac{\text{the F or O or P amounts entrapped in niosomes} \times 100}{\text{the total F or O or P amounts in the niosomal dispersion}}$$

Preparation of Gel and Cream Containing Niosomes Entrapped with the Semi-purified Extracts

Descriptions, codes, and compositions of all samples were listed in Table I. Gel nio was prepared by dispersing Carbopol 980 in the niosomal dispersion, then gently stirred by a magnetic stirrer until homogenous. The mineral oil and concentrated (conc.) paraben (the mixture of 18% *w*/*v* of methyl paraben and 2% *w*/*v* of propyl paraben in propylene glycol) at 1% *w*/*w* were added into the dispersion with continuous mixing until homogeneous and then triethanolamine was added with vigorously mixing until the homogeneous gel was obtained. For Cream nio, oil phase containing cetyl alcohol (m.p. 49°C), white petrolatum (m.p. 38°C), mineral oil, and isopropyl myristate was heated above their melting point at 65± 2°C. A mixture of niosomal dispersion and the aqueous phase containing Carbopol 980, glycerin, propylene glycol except the conc. paraben was heated at 65±2°C. The temperature of the oil and aqueous phase was maintained at 65±2°C. Then, the oil

Table I. Descriptions, Codes, and Compositions of the Systems for Transdermal Absorption Through Excised Rat Skin by Franz Diffusion Cells

Code of the systems	Description	Compositions
Mixed FOP	Mixtures of the F, O, and P extracts	The rice bran extract mixtures containing 0.5% F, 1.5% O, and 1.5% P
Nio FOP	Niosomes entrapped with the extracts containing F, O, and P	Niosomes entrapped with the rice bran extracts containing 0.5% F, 1.5% O, and 1.5% P
Gel FOP	Gel incorporated with the extracts containing F, O, and P	The rice bran extract mixtures containing 0.5% F, 1.5% O, and 1.5% P
Cream FOP	Cream incorporated with the extracts containing F, O, and P	The rice bran extract mixtures containing 0.5% F, 1.5% O, and 1.5% P
Gel nio	Gel incorporated with niosomes entrapped with the extracts containing F, O, and P	97% of niosomes entrapped with the rice bran extracts containing 0.5% F, 1.5% O, and 1.5% P
Cream nio	Cream incorporated with niosomes entrapped with the extracts containing F, O, and P	85% of niosomes entrapped with the rice bran extracts containing 0.5% F, 1.5% O, and 1.5% P

phase was poured into the aqueous phase and stirred until the mixture was cooled to 40°C. The triethanolamine and conc. paraben solution were added into the mixture and stirred continuously. Gel FOP and Cream FOP were prepared by the same procedure as Gel nio and Cream nio, respectively, but distilled water was used instead of the niosomal dispersion. The semi-purified extracts containing F and P were added in the aqueous phase, while the extract containing O was added into the oil phase.

Transdermal Absorption

The animal experiment was conducted in full compliance with ethical principles with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use. This experiment has been approved by the Animal Ethics committee at Faculty of Medicine, Chiang Mai University in Thailand and performed at Faculty of Pharmacy, Chiang Mai University in Chiang Mai, Thailand. The rats obtained from the National Laboratory Animal Centre, Mahidol University in Nakhon Pathom, Thailand, were shipped to Chiang Mai University by DHL. The transdermal experiment of six samples listed in Table I has been investigated by vertical Franz diffusion cells and the cells were stop at different time intervals of 1, 3, and 6 h. All experiments were performed in triplicate. The hair on the abdominal area of the rats was shaved off and left overnight. The rats were sacrificed. The abdominal skin of one rat was cut into three pieces for one sample at each experiment. Hence, the skin from the identical rat for each sample was used at different time intervals. Thus, the total number of the male Sprague–Dawley rats (weight range, 200–250 g) used in this study was 18. Then, the subcutaneous fat was trimmed off. The rat skin was mounted on the Franz diffusion cell with the surface of 2.46 cm^2 and the receiving compartment volume of 13.5 ml with the dermal side of the skin exposed to the receiving solution and the SC side remained in contact with the donor compartment. The receiving solution was the phosphate-buffered saline solution (pH 7.4), controlled at 37.0±2.0°C, and constantly stirred at 100 rpm with a small magnetic bar throughout the experiment. An amount of 0.50 g samples was placed into the donor compartment and covered with paraffin film. After 1, 3, and 6 h, the diffusion cell setup was dismantled. The rat skin was removed from the cell and swung twice in 100 ml of DI water and the rinsed water was discarded. The receiving solution was withdrawn and freeze-dried at −52°C and 0.05 mbar for 24 h in a freeze-dryer (CHRIST, Martin Christ, Germany). Then, 1 ml of absolute ethanol was added to the freeze-dried powder and determined for the contents of F and O by HPLC, while 1 ml of distilled water was added to the powder for the analysis of P by spectrophotometer. The F, O, and P contents in SC were collected by the stripping method as previously described (24). Briefly, the skin was stripped with 20 pieces of the adhesive tapes (size = 15×20 mm; 3 M Scotch MagicTM tape). Each tape with the sufficient size to cover the full area of the skin was put in contact with the treated skin. The tape on the skin was charged with the weight of 200 g for 10 s and thereafter removed rapidly. The tapes were then pooled, put in 10 ml of ethanol (for F and O) or 10 ml of distilled water (for P) and sonicated for 1 min. The mixture was filtered and the filtrate was dried by a freeze drier and assayed for F and O by HPLC and P by spectrophotometer as previously described (6). The cumulative amount of F, O, and P that diffused through the skin by unit area (ng/cm^2) was plotted as the function of time for each sample. All experiments were performed in triplicate. Data were expressed as the mean ($\bar{x}$) of the three experiments ± the standard deviation (SD) and were analyzed using ANOVA with LSD test. Statistical analysis differences yielding $p<0.05$ were considered significant.

RESULTS

The F and O contents in the semi-purified extracts determined by HPLC were 0.289±0.022 and 14.41±0.69 mg/g, respectively. P content in the semi-purified extracts determined by a spectrophotometer was 10.47±0.28 mg/g. The blank niosomes composed of Tween 61 mixed with cholesterol at 1:1 molar ratio which gave the highest physical stability at 4, 30, and 45°C for 3 months, were selected to entrap the semi-purified extracts containing F, O, and P at the maximum loading of 0.5%, 1.5%, and 1.5%, respectively (data not shown).

Characteristics of Niosomes Entrapped with the Semi-purified Extracts Containing F, O, and P

Vesicular Size and Morphology

The mean vesicular sizes of blank niosomes and niosomes entrapped with the semi-purified extracts observed by dynamic light scattering were 566.8±411.5 and 480.9±270.8 nm, respectively. Figure 1 showed the freeze fracture TEM images of the niosomes entrapped and not entrapped (blank) with the semi-purified extracts containing F, O, and P, and niosomes incorporated in gel and cream formulations. The morphology of the entrapped and unentrapped (blank) niosomes was the mixture of large unilamellar vesicles (LUVs) and multivesicular vesicles.

Transition Temperature Analysis

Niosomes entrapped and not entrapped with the semi-purified extracts containing F, O, and P gave no significant different phase transition temperatures (77±3 and 80±4°C; $p<0.05$, paired *t* test; Fig. 2).

Niosomal Membrane Rigidity Analysis

For the vesicular membrane rigidity measurement, the niosomal dispersion was incubated with DPH. Then, the rate of DPH incorporation into the bilayer region of the niosomes was monitored by recording the fluorescence of DPH emission at 450 nm following excitation at 350 nm. The decrease in fluorescence intensity could be associated with the decrease of the packing structure of niosomes. In Fig. 3, the fluorescence polarization of DPH in the vesicular membranes of blank niosomes and niosomes entrapped with the semi-purified extracts containing F, O, and P (Nio FOP) gradually decreased and became constant with increasing temperatures. This indicated that the vesicular membrane of niosomes was flexible at high temperature.

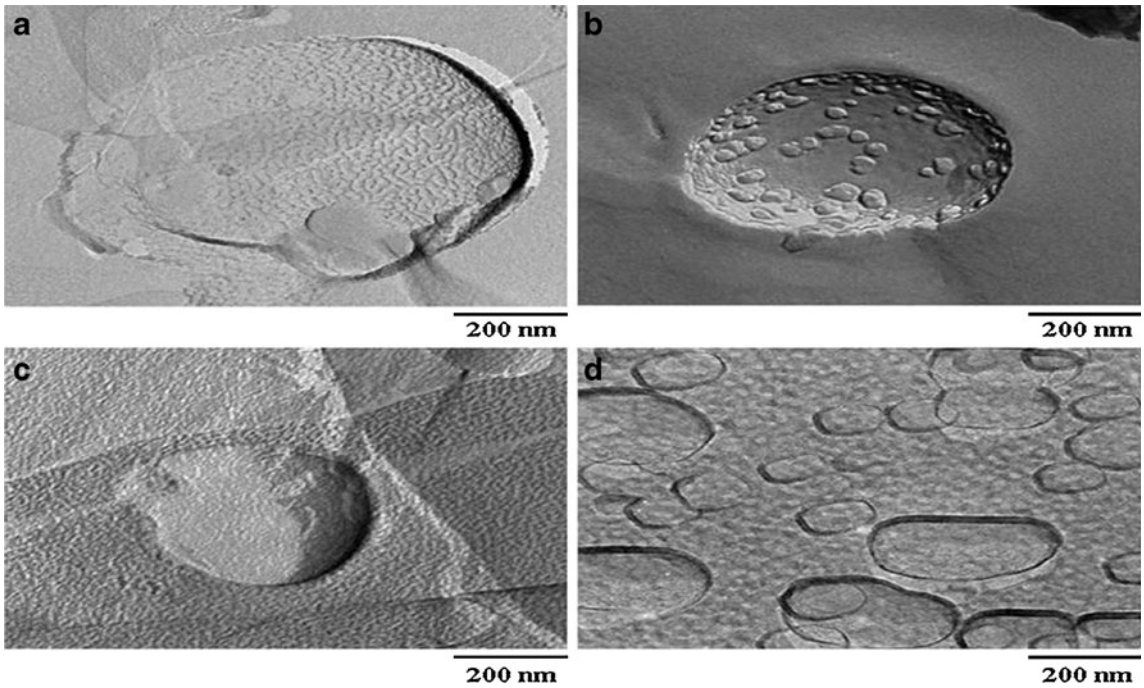

Fig. 1. Freeze fracture TEM images of niosomes composed of Tween 61 mixed with cholesterol (20 mM) at 1:1 molar ratio **a** blank niosomes prepared by $scCO_2$ technique; **b** niosomes entrapped with the semi-purified mixed extracts containing F, O, and P; **c** niosomes entrapped with the extracts incorporated in gel formulation; and **d** niosomes entrapped with the extracts incorporated in cream formulation (magnification ×12.0k, *scale bar* 200 nm)

Entrapment Efficiency Determination

The entrapment efficiencies of F (60.21±0.02%) and P (49.83±0.98%) which are polar compounds were higher than O (45.91±1.010%) which is a hydrophobic molecule of 1.3 and 1.1 times, respectively.

Transdermal Absorption of F, O, and P Through Excised Rat Skin

The cumulative amount (ng/cm^2) in the rat skin at 1, 3, and 6 h of F, O, and P was shown in Figs. 4, 5, and 6, respectively. At all time intervals, F and O from all samples except Gel nio and P from all systems, were found in the receiving solution.

Transdermal Absorption of F from Various Systems at 6 h

For Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of F in SC, VED, and receiving solution (0.228±0.006, 0.145±0.025, and 0.205±0.019 ng/cm^2, respectively) than those from Nio FOP (0.120±0.013, 0.131±0.006, and 0.002±0.0 ng/cm^2, respectively) of 1.9, 1.1, and 102.5 times. For Gel FOP and Gel nio, Gel FOP gave higher cumulative amount of F in SC (1.025±0.008 ng/cm^2) than that from Gel nio (0.416±0.010 ng/cm^2) of 2.5 times. Moreover, Gel FOP gave the cumulative amount of F in receiving solution at 0.014±0.0004 ng/cm^2, while F from Gel nio was not observed in receiving solution. On the contrary, the cumulative amount of F in VED from Gel FOP (0.276±0.062 ng/cm^2) was lower than that from Gel nio (1.564±0.052 ng/cm^2) of 5.7 times. For Cream FOP and Cream Nio, Cream FOP gave higher cumulative amounts of F in VED and receiving solution (0.947±0.025 and 0.591±0.002 ng/cm^2, respectively) than those from Cream nio (0.680±0.049 and 0.182±0.017 ng/cm^2, respectively) of 1.4 and 3.2 times. The cumulative amount of F in SC from Cream FOP (0.470±0.012 ng/cm^2) was lower than that from Cream nio (0.617±0.036 ng/cm^2) of 1.3 times. Moreover, Cream FOP gave higher cumulative amounts of F in SC, VED, and receiving solution (0.470±0.012, 0.947±0.025, and 0.591±0.002 ng/cm^2, respectively) in comparing to those from Mixed FOP (0.228±0.006, 0.145±0.025, and 0.205±0.019 ng/cm^2, respectively) of 2.1, 6.5, and 2.9 times, respectively.

Transdermal Absorption of O from Various Systems at 6 h

For Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of O in SC and receiving solution (6.585±0.573 and 9.084±0.093 ng/cm^2, respectively) than from those from Nio FOP (5.065±0.082 and 1.255±0.135 ng/cm^2, respectively) of 1.3 and 7.2 times, respectively. On the other hand, Nio FOP showed higher cumulative amount of O in VED (12.630±0.445 ng/cm^2) than the unentrapped O (5.295±0.172 ng/cm^2) of 2.4 times. For Gel FOP and Gel nio, Gel FOP gave higher cumulative amounts of O in SC and VED (7.023±0.356 and 2.201±0.350 ng/cm^2, respectively) than those from Gel nio (0.979±0.121 and 1.426±0.140 ng/cm^2, respectively) of 7.2 and 1.5 times, respectively. Moreover, Gel FOP gave cumulative amount of O in receiving solution at 0.449±0.205 ng/cm^2, while O from Gel nio was not observed in the receiving solution. For Cream FOP and Cream nio, Cream FOP gave higher cumulative amounts of O in SC and receiving solution (5.723±0.462 and 1.862±0.106 ng/cm^2, respectively) than those from the Cream nio (3.106±2.132 and 1.103±0.082 ng/cm^2, respectively) of 1.8 and 1.7 times, respectively, while the cumulative amount of O in VED from Cream FOP (0.518±0.182 ng/cm^2) was lower than that from Cream nio (15.972±0.273 ng/cm^2) of 31 times.

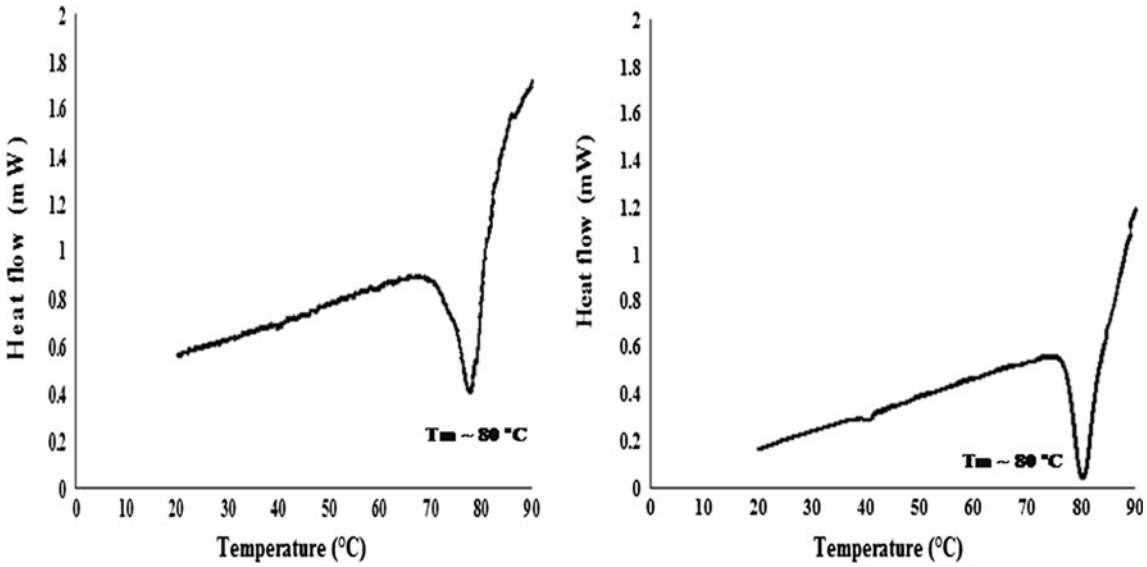

Fig. 2. DSC curves of **a** blank niosomes and **b** niosome entrapped with the semi-purified mixed extracts containing F, O, and P prepared by $scCO_2$ technique

Transdermal Absorption of P from Various Systems at 6 h

For Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of P in SC and VED (1.211±0 and 25.857±0.025 ng/cm^2, respectively) than those from Nio FOP (0.119±0.0 and 15.979±0.030 ng/cm^2, respectively) of 10 and 1.6 times, respectively. However, P from both Mixed FOP and Nio FOP could not be found in the receiving solution. For Gel FOP and Gel nio, Gel FOP gave no significant different cumulative amounts of P in SC and VED (1.381±0.008 and 7.447±0.045 ng/cm^2, respectively) in comparing to those from Gel nio (1.336±0.075 and 7.365±0.014 ng/cm^2, respectively). However, these two gel formulations gave higher cumulative amount of P in SC (1.381±0.008 and 1.336±0.075 ng/cm^2, respectively) than that from Mixed FOP (1.211±0 ng/cm^2) of 1.1 times. On the contrary, the cumulative amount of P in VED from these two gel formulations (7.447±0.045 and 7.365±0.014 ng/cm^2, respectively) was lower than that from Mixed FOP (25.857±0.025 ng/cm^2) of 3.5 times. P from these two gel formulations was not observed in the receiving solution. For Cream FOP and Cream nio, Cream FOP gave higher cumulative amount of P in SC (1.650±0.001 ng/cm^2) than that from the Cream nio (1.420±0.050 ng/cm^2) of 1.2 times, while the cumulative amount of P in VED from Cream FOP (10.708±0.018 ng/cm^2) was lower than that from Cream nio (13.753±0.03 ng/cm^2) of 1.3 times. These two cream formulations gave higher cumulative amount of P in SC (1.650±0.001 and 1.420±0.050 ng/cm^2, respectively) than that from Mixed FOP (1.211±0 ng/cm^2) of 1.4 and 1.2 times, respectively, whereas the cumulative amount of P in VED from these two cream formulations (10.708±0.018 and 13.753±0.030 ng/cm^2, respectively) was lower than that from Mixed FOP (25.857±0.025 ng/cm^2) of 2.4 and 1.9 times, respectively. P could not be found in the receiving solution from these two cream formulations. No P from all systems was found in the receiving solution.

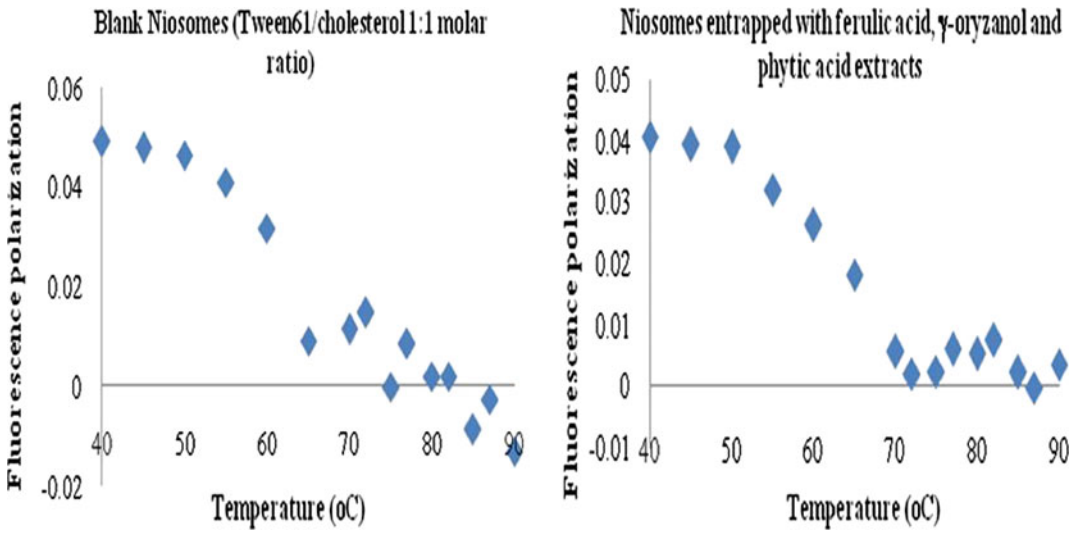

Fig. 3. The relationship between temperatures and fluorescence polarization of **a** blank niosomes and **b** niosome entrapped with the semi-purified mixed extracts containing F, O, and P prepared by $scCO_2$ technique

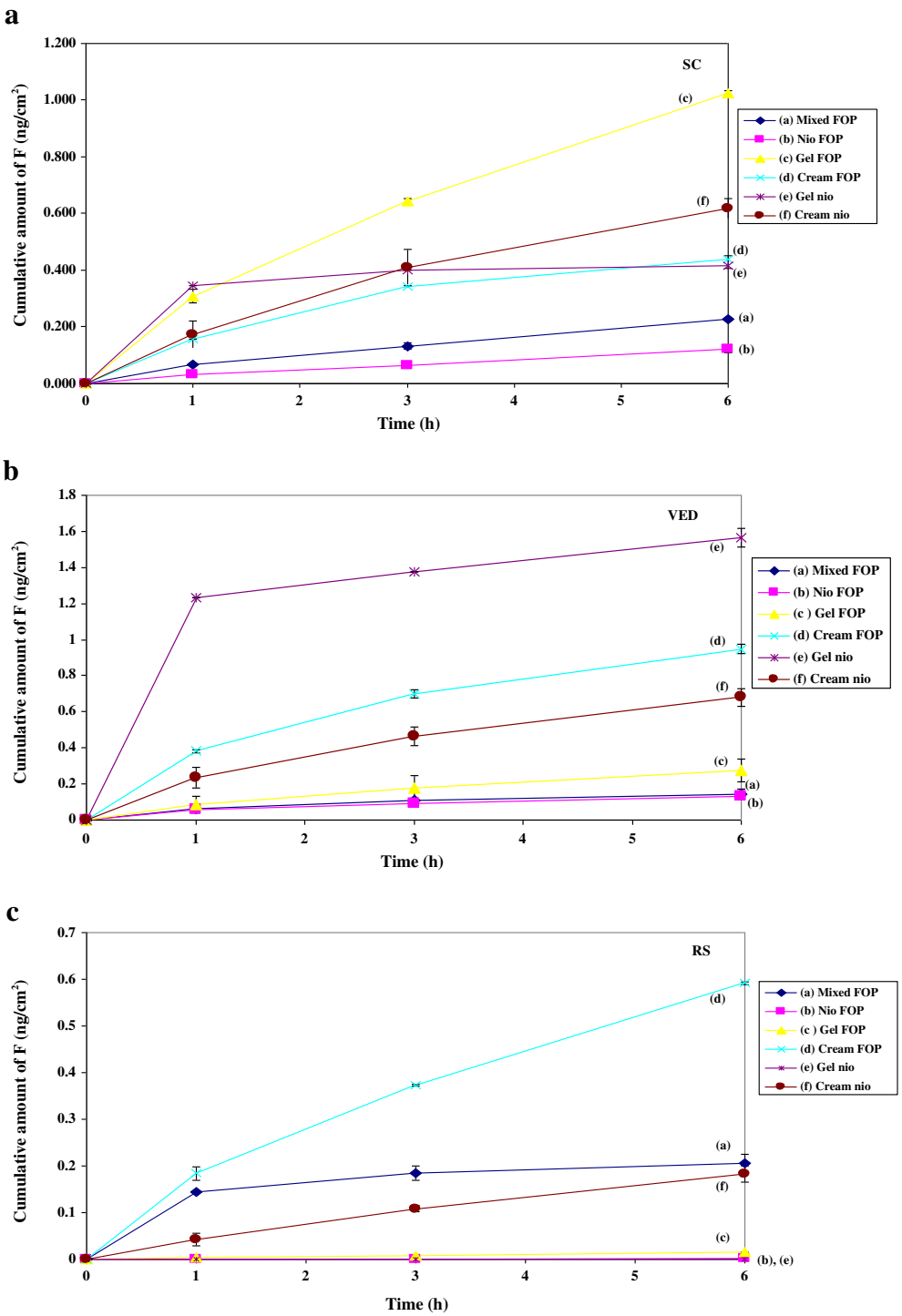

Fig. 4. The cumulative amount (nanograms per square centimeter) of F at 1, 3, and 6 h from various formulations in **a** stratum corneum (*SC*), **b** viable epidermis and dermis (*VED*), and **c** receiving solution (*RS*) by vertical Franz diffusion cells. Each value represents mean±SD and $n=3$

DISCUSSION

The rice bran semi-purified extracts are the crude extracts of the rice bran which have been partially purified to contain more amounts of the bioactive compounds F, O, and P than the crude extracts. Three semi-purified extracts containing 0.29±0.022, 14.41±0.690, and 10.47±0.280 mg/g, of F, O, and P, respectively, were used in this study. The maximum loading in niosomes was at 0.5%, 1.5%, and 1.5% *w*/*w* of F, O, and P, respectively. The maximum loading of F containing in the semi-purified extract was less than O and P because the extract contained less amount of F than O and P. Also, the standards of

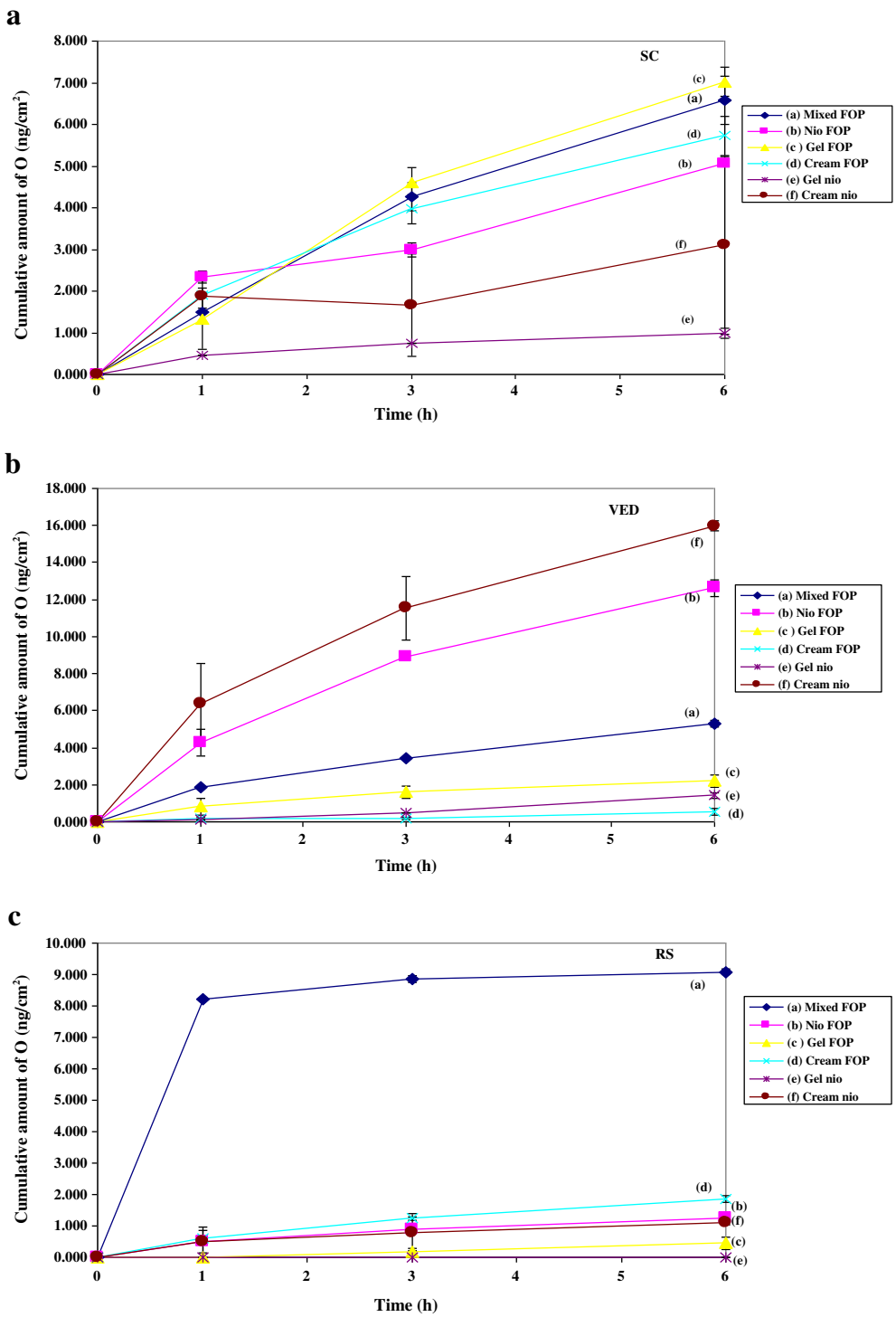

Fig. 5. The cumulative amount (nanograms per square centimeter) of O at 1, 3, and 6 h from various formulations in **a** stratum corneum (*SC*), **b** viable epidermis and dermis (*VED*), and **c** receiving solution (*RS*) by vertical Franz diffusion cells. Each value represents mean±SD and $n=3$

the semi-purified rice bran extracts are important for the reproducing experiment. The degrees of purifications have been set as the standard for the semi-purified F, O, and P extracts. It was calculated from the ratio of F, O, and P contents in the semi-purified extracts to the F, O, and P contents in the crude extracts (25), which were 1.9-, 1.6-, and 4.6-fold of the crude extracts, respectively.

Characteristics of Niosomes Entrapped with the Semi-purified Extracts Containing F, O, and P

Vesicular Size and Morphology

The mean vesicular size of niosomes entrapped with the extracts was slightly smaller than the blank niosomes of about

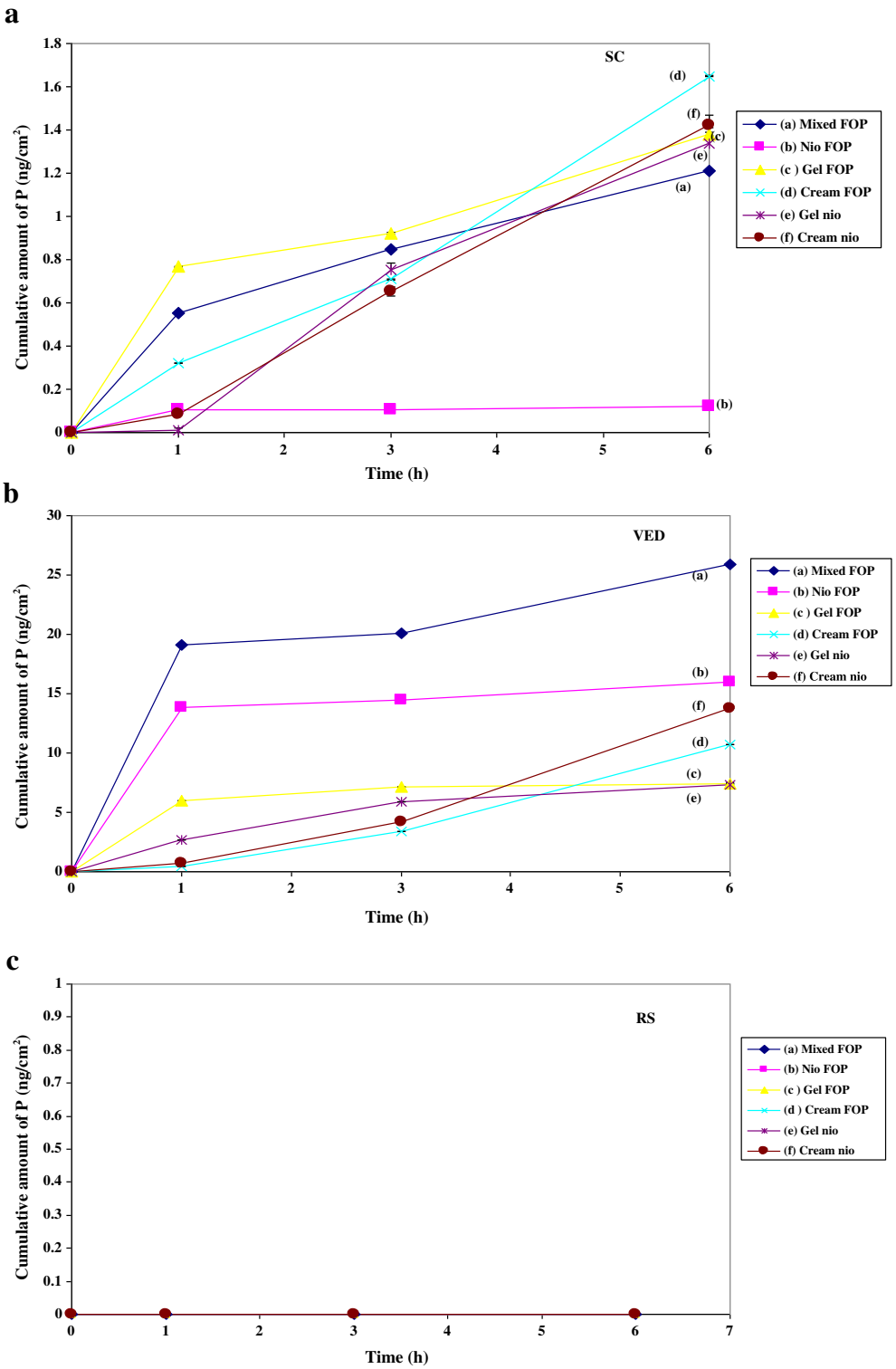

Fig. 6. The cumulative amount (nanograms per square centimeter) of P at 1, 3, and 6 h from various formulations in **a** stratum corneum (*SC*), **b** viable epidermis and dermis (*VED*), and **c** receiving solution (*RS*) by vertical Franz diffusion cells. Each value represents mean±SD and $n=3$

1.18 times. Tween 61 is non-ionic in nature. But, the hydroxyl moiety of the cholesterol which is in the niosomal compositions can give the negative nature of the niosomes (26). Thus, the charges of the entrapped compounds can affect the characteristics of our niosomes (27). The entrapped phytic acid (P), which has the hydrophilic nature and the positive charge in the aqueous phase between the bilayers of the niosomes, may also adsorb on and neutralize the negative charges of the niosomal membrane thereby reducing the repulsion between the bilayers, giving the smaller aqueous spaces and the smaller vesicular sizes (19,28,29).

The size distribution of all niosomes exhibited a broad distribution with slightly large, but still in the nanosize range. Thus, the transdermal delivery of F, O, and P entrapped in the niosomes can still be expected. In fact, the more narrow size distribution of niosomes prepared by the $scCO_2$ technique can be improved using ultrasonication (30) or polycarbonate extrusion method (31). The entrapped and unentrapped (blank) niosomes were in large unilamellar vesicles and multivesicular vesicles. The mechanism of vesicular formation by the $scCO_2$ technique was similar to the reverse phase evaporation method that yielded the LUVs and multivesicular vesicles with the sizes ranging from 0.1 to 1 μm (20). According to the results of the transition temperature analysis, niosomes entrapped with the semi-purified extracts containing F, O, and P showed the phase transition temperatures at about 80°C. This indicated that the niosomal dispersion was thermal stable at up to 80°C. However, the mechanical mixing in the incorporation process of niosomes in gel and cream formulations might slightly reduce the particle size as observed from the TEM image, but not significantly different compared with the initial. The size changes of niosomes incorporated in gel and cream formulations by light scattering were not performed since there were many unknown particles in gel and cream such as oil droplets which might interfere with the measurement. Nevertheless, niosomes in gel and cream formulations could still be observed by FF-TEM. This has confirmed the existing of niosomes after incorporated in gel and cream formulations.

Transition Temperature Analysis

Differential scanning calorimetry (DSC) was performed to investigate gel–liquid transition temperature of niosomes. The phase transition temperature is the temperature that induces a changing of lipid physical state from gel phase (closely packed molecule) to liquid crystalline phase (loosely packed molecule and fluid). Distilled water was used as the reference. During each scanning temperature, the heat capacity difference between niosomes and distilled water was plotted as the function of temperature. Thus, the scanning temperature should not be reached the boiling point of water (100°C). In fact, the phase transition temperature of nanovesicles dispersed in water has been previously reported to be between 10°C to 90°C (21,22). Niosomes entrapped and not entrapped with the semi-purified extracts containing F, O, and P gave no significant different phase transition temperatures (77±3°C and 80±4°C; $p<0.05$, paired t test). This result has suggested that the extracts containing F, O, and P do not interfere with the formation of niosomes (32).

Niosomal Membrane Rigidity Analysis

As known, the niosomal membrane rigidity indicating the information of packing structure of the vesicular membrane is directly correlated to the fluorescence polarization. The fluorescence polarization of DPH in the vesicular membranes of blank niosomes and niosome entrapped with the semi-purified extracts containing F, O, and P (Nio FOP) gradually decreased and became constant with increasing temperature. This might be due to the intercalation of cholesterol between the non-ionic surfactants (Tween 61) which stabilized the niosomal membrane (33). Moreover, the temperature dependence of membrane rigidity of Nio FOP was quite similar to that of the blank niosomes. Thus, the semi-purified extracts containing F, O, and P did not appear to influence the rigidity of niosomal membranes thereby not interfering with the process of niosomal formation.

Entrapment Efficiency Determination

The entrapment efficiencies of the bioactive compounds in the bilayer vesicles depend not only on the polarity and molecular weight or size of the compounds, but also the lamellarity and sizes of the niosomes. The entrapment efficiencies of F (60.21± 0.020%) and P (49.83±0.980%) in niosomes which were the mixture of LUV and multivesicular structure were higher than O (45.91±1.010%) of 1.3 and 1.1 times, respectively, because of the high aqueous spaces that could entrap more hydrophilic compounds. The low entrapment efficiency of O in the LUV niosomes was due to the less lipid bilayers (15,34). Also, the large molecular structure of O (M.W. of 602.9 $gmol^{-1}$) might be less intercalated between the bilayer (35). For the entrapment process, F, O, and P were entrapped in niosomes by introducing CO_2 into the stirred-view cell containing a mixture of Tween 61, cholesterol, and the semi-purified extract containing F, O, and P in distilled water. Then, pressure and temperature in the system were increased to 200 bar and 60± 1°C to produce supercritical carbon dioxide fluid ($scCO_2$). In this step, the inside of the viewed cell became opaque due to the formation of CO_2-in-water (CO_2/W) emulsion and Tween 61, cholesterol together with O was dissolved in the formation of CO_2/W owing to the high solvating power of $scCO_2$. During the depressurization, the mixture of LUV and multivesicular niosomes would be formed in water through the phase inversion from CO_2/W emulsion to niosomes (36). F and P were more passive entrapped into niosomes due to the driving force of the saturated non-ionized F and P in carbonic water. The achieving high entrapment efficiency of hydrophilic F and P in niosomes by $scCO_2$ was from not only the acidic pH in the system, but also the high aqueous spaces of large unilamellar niosomes (37–39).

Transdermal Absorption Study

Transdermal Absorption of F from Various Systems at 6 h. In comparing Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of F in SC, VED, and receiving solution than those from Nio FOP. The skin penetration of F decreased when entrapped in niosomes. The vesicular size of Nio FOP (480.9±270.8 nm) was larger than the unentrapped F (Mixed FOP), might retard the penetration of F (40,41). In comparing Gel FOP and Gel nio, Gel FOP gave higher cumulative amounts of F in SC and receiving solution than those from Gel nio. The gel structure may promote the penetration of F across SC owing to the occlusion effects from the oil and fat compositions which can enhance skin hydration (42,43). On the contrary, the cumulative amount of F in VED from Gel FOP was lower than that from Gel nio. Niosomes may interfere with the membrane structure of SC and directly fuse with the upper layer of the skin, thereby enhancing the skin permeation and accumulation of F in the VED (44,45). Moreover, the penetration of F from Gel nio was not observed in the receiving solution, but was found from Gel FOP. The large vesicular size of niosomes incorporated in gel formulation may lower the penetration of F through the skin. Thus, niosomes can provide not only a high intradermal accumulation reservoir for F, but also

reduce the systemic effects of F. In comparing Cream FOP and Cream Nio, Cream FOP gave higher cumulative amounts of F in VED and receiving solution than those from Cream nio. Moreover, Cream FOP gave higher cumulative amounts of F in SC, VED, and receiving solution in comparing to those from Mixed FOP. Some penetration enhancers in the cream compositions such as glycerin may modify the solubility of F (46,47) and promote the penetration of F across the SC. However, the cumulative amount of F in SC from Cream FOP was lower than that from Cream nio. The structure of niosomes in the cream formulation may retard the diffusion of F from the inner aqueous phase of niosomes through SC, thereby prolonging the penetration of F (48). Therefore, niosomes incorporated in gel (Gel nio) was the most effective formulation for transdermal delivery of F due to the higher cumulative amount of F in VED than that from Mixed FOP, Nio FOP, Gel FOP, Cream FOP, and Cream Nio of 10.9, 11.9, 5.7, 1.6, and 2.3 times, respectively, while no F from Gel nio was found in the receiving solution indicating of less systemic risk.

Transdermal Absorption of O from Various Systems at 6 h. In comparing Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of O in SC and receiving solution than those from Nio FOP, while the penetration of O in VED from Mixed FOP was lower than that from Nio FOP. The high lipophilicity of O may facilitate its partition to the upper layers of the SC and further diffuse into the deeper skin. On the other hand, Nio FOP showed higher cumulative amount of O in VED than the unentrapped O. When niosomes contacted with the skin, the non-ionic surfactants in the niosomal membrane might disrupt the membrane structure of SC and accelerate the permeation of O into the deeper skin layer (49). In comparing Gel FOP and Gel nio, Gel FOP gave higher cumulative amounts of O in SC, VED, and receiving solution than those from Gel nio. O, a hydrophobic compound, can be solubilized and assemble into the vesicular membrane. Hence, the partitioning of O between the niosomal membrane and the hydrophilic crosslink matrix of the gel is expected to be reduced resulting in the prolonged release. In comparing Cream FOP and Cream nio, Cream FOP gave higher cumulative amounts of O in SC and receiving solution than those from the Cream nio, while the cumulative amount of O in VED from Cream FOP was lower than that from Cream nio. Therefore, when entrapped in niosomes and incorporated in the cream formulation, the penetration and accumulation of O in the deeper skin layer were increased. The structure of niosomes may provide the saturation of O at the interface between the niosomal membrane and the cream that may enhance the diffusion of O into the skin. However, Cream FOP showed higher cumulative amount of O in the receiving solution than that from Cream nio. This has confirmed the retarding effects of O on skin permeation from niosomes when incorporated in cream formulations. Cream nio gave higher cumulative amount of O in VED than that from Mixed FOP, Nio FOP, Gel FOP, Gel Nio, and Cream FOP of 3, 1.3, 7.2, 11.2, and 31 times, respectively. Moreover, O from Gel nio was not found in the receiving solution the same as that of F, indicating of less systemic risk. This result has indicated that niosomes incorporated in cream (Cream nio) appear to be the most effective formulation for transdermal delivery of O.

Transdermal Absorption of P from Various Systems at 6 h. In comparing Mixed FOP and Nio FOP, Mixed FOP gave higher cumulative amounts of P in SC and VED than those from Nio FOP. The larger vesicular size of Nio FOP than the unentrapped P may reduce the penetration across the SC and subsequently lower the cumulative amount of P in the deeper skin layer the same as that of F. However, P from both Mixed FOP and Nio FOP could not be found in the receiving solution. This was due to the high hydrophilicity and large molecular size of P. In comparing Gel FOP and Gel nio, Gel FOP gave no significant different cumulative amounts of P in SC and VED in comparing to those from the Gel nio. However, these two gel formulations gave higher cumulative amount of P in SC than that from Mixed FOP due to the occlusion effects of the gel and the permeation enhancement of the niosomes. On the contrary, the cumulative amount of P in VED from these two gel formulations was lower than that from Mixed FOP. Also, P from these two gel formulations was not observed in the receiving solution. This might be not only from the high hydrophilicity, ionic property, and molecular structure of P, but also the large vesicular size of niosomes that might retard the penetration of P through the skin. In comparing Cream FOP and Cream nio, Cream nio gave higher cumulative amount of P in VED than that from Cream FOP. This has confirmed the enhancing effect of niosomes on skin penetration of P when incorporated in cream formulation. However, these two cream formulations gave lower cumulative amount of P in VED than that from Mixed FOP. Also, P could not be found in the receiving solution from these two cream formulations. The large vesicular size of niosomes and some polar constituents in the cream formulation may bind with P and facilitate the accumulation of P in the skin. Thus, Mixed FOP showed the highest cumulative amount of P in VED in comparing to that from Nio FOP, Gel FOP, Gel nio, Cream FOP, and Cream nio of 1.6, 3.5, 3.5, 2.4, and 1.9 times, respectively.

Transdermal absorption of F, O, and P through excised rat skin. At 6 h, F and P from Nio FOP gave lower cumulative amount in the skin (VED) than that from Mixed FOP, while O from Nio FOP exhibited higher cumulative amount in VED than that from Mixed FOP. This study has indicated that niosomes enhance transdermal absorption of the lipophilic compound (O) due to its fusion capability with the skin, but retard the hydrophilic compounds (F and P) owing to their large molecular sizes and the multilayers of niosomes (41,50–52). This has also suggested the less systemic effects of F and P than O. Niosomes may affect the transdermal absorption of the entrapped compound via several mechanisms (41). For the lipophilic O which intercalated between the niosomal membrane, the fusion between the skin structure and niosomes was the major factor for driving O through the skin (49). For the hydrophilic F and P, the vesicle sizes of niosomes were the major factor that affected the transdermal absorption of these compounds (40,41). Although both F and P have a similar hydrophilicity, the penetration of P was retarded by its larger molecular structure.

In summary, the semi-purified extracts of 3.5% *w*/*w* containing F, O, and P at 0.289±0.022, 14.41±0.69, and 10.47±0.28 mg/g, respectively, were entrapped in niosomes. The maximum loading in niosomes was 0.5%, 1.5%, and 1.5% *w*/*w* for F, O, and P, respectively, in which the total content of the three components was not higher than 2.5%. Since the amount of the samples used to carry out transdermal absorption was 0.50 g or ml, the amounts of F, O, and P in this study were 0.0007, 0.11, and 0.08 mg, respectively. Although the weight percentages of F, O,

and P were in small amount, the potential of niosomes in facilitating the transdermal penetration through rat skin of these active compounds was observed. Generally, the unionized molecules are more permeable through the skin by the lipid pathway due to their high distribution in the oily phase, whereas the ionized molecules can permeate through the skin by skin pore—minor pathway (53,54). Thus, O would pass easily through the skin due to its unionized molecule and high hydrophobicity, while the skin permeations of acidic F and P compounds are a more complex process due to the effect of pH to produce the ionized or non-ionized molecules. In fact, skin permeation of the acidic compounds should be higher at lower pH. However, some literatures have reported that there was no influence of pH of the vehicle on skin permeation due to the high aqueous solubility or saturation of the acidic compounds at high pH that may force more molecules to permeate through the skin, thereby balancing the decreased penetration of the non-ionized species (55,56). Thus, no matter at low or high pH, the total penetration depended on the lipophilicity of the molecules since it could not be overcome by the increased concentration of the ionized species (57).

CONCLUSION

Niosomes composed of Tween 61 mixed with cholesterol entrapped and not entrapped with the semi-purified rice bran extracts containing F, O, and P prepared by the supercritical carbon dioxide ($scCO_2$) were in the mixture of large unilamellar and multivesicular vesicular structures with the average size of 480.9±270.8 and 566.8±411.5 nm, respectively. The phase transition temperature of niosomes entrapped and not entrapped with the extracts were approximately 80°C demonstrating that the rice bran extracts did not interfere with the formation of niosomes. The entrapment efficiencies of the rice bran hydrophilic bioactive compounds (F and P) in niosomes were 60.21±0.02% and 49.83±0.98%, respectively, which were about 1.3 and 1.1 times more than that of the lipophilic compound (O; 45.91±1.01%). Transdermal absorption through rat skin at 6 h revealed that F and P from Nio FOP gave lower cumulative amount in the skin (VED) than that from Mixed FOP of 1.1 and 1.6 times, respectively, while O from Nio FOP exhibited higher cumulative amount in VED than that from Mixed FOP of 2.4 times. Niosomes entrapped with F, O, and P incorporating in gel (Gel nio) and cream (Cream nio), and the mixture of the semi-purified extracts containing F, O, and P (Mixed FOP) exhibited the highest cumulative amounts of F, O, and P through the rat skin at 1.564, 15.972, and 25.857 ng/cm^2, respectively, indicating the effects of niosomes, bioactive lipophilicity, and formulation types on transdermal absorption of the bioactive compounds.

ACKNOWLEDGMENTS

This work was supported by the Thailand Research Fund (TRF) under the RGJ-PhD program, Natural Product Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), and Nanoscience and Nanotechnology Research Center Project, Faculty of Sciences, Chiang Mai University, Thailand.

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