INTRODUCTION

Retinyl palmitate (RP) is an ester form of retinol (vitamin A) and palmitic acid. RP can be hydrolyzed to retinol after enzymatic cleavage of the ester bond, and further metabolized to retinoic acid which has a pharmacological effect. RP is widely used as an active ingredient in pharmaceutical and cosmetic products. RP is known as a potent anti-aging agent for the prevention and treatment of wrinkles (Boehnlein et al., 1994; Jee et al., 2006; Ro et al., 2013).

The considerable interest in beauty and eternal youth with minimal wrinkles which are one of the most typical signs of the aged skin has led to a large market for cosmetics exerting anti-wrinkle effects (Pena et al., 2010). Photo-aged skin exhibits alterations to the dermal extracellular matrix (ECM) such as deposition of elastic fibers and decreased levels of collagen (Watson et al., 2008). RP can protect the skin against skin-aging by neutralizing unstable free radicals, increasing the fibroblasts, and participating in the process of collagen propagation in the dermis (Kim et al., 2003; Jee et al., 2006).

Although RP is thermally more stable than retinol (Idson, 1990), it is still problematic in terms of stability since it has been shown that RP is easily oxidized than the parent compound, retinol. Retinoids usually present low permeation into the skin, because most of the drug is broken before percutaneous absorption or still remains on the surface due to stratum corneum (SC) known as an effective barrier (Ihara et al., 1999; Carlotti et al., 2002; Carlotti et al., 2004; Teixeira et al., 2010).

Therefore, several approaches for stabilizing RP have been suggested; nanocapsules, solid lipid nanoparticles, microcapsules and liposomes. However, these earlier efforts on stabilizing RP focused on protecting RP from photo oxidation by blocking the UV light (Carlotti et al., 2004; Carlotti et al., 2005; Sane and Limtrakul, 2009). Thus, we investigated polysaccharides as a new class of stabilizer for RP in our previous study.
and clearly found that pectin has a considerably stronger anti-
oxidative activity than any other polysaccharides we exam-
ined so that can improve the stability of RP (Ro et al., 2013).
The advantages of employing polysaccharides as a stabilizer
over the particulate systems of RP are considered to be safe,
availability, and possibility of developing various dosage forms
such as gels, creams, films as well as nano- and micro-encap-
sulated skin delivery systems.

Many attempts have been made to improve the penetra-
tion of drugs across the skin with a variety of enhancement
mechanisms such as increase in the solubility of a drug within
SC (e.g., transcutol) and disruption of SC structure (e.g., ter-
penes) (Harrison et al., 1996; Jain et al., 2002). However, little
has been known whether stabilizing agents can modulate the
skin penetration properties. Therefore, the aim of the current
study was to examine the effect of the stabilization of RP on its
permeation and distribution characteristics by using the
franz diffusion cell (Jenning et al., 2000; Antille et al., 2004).
Pectin and ascorbyl palmitate were used as anti-oxidative sta-
bilizers for RP.

MATERIALS AND METHODS

Materials

Pectin from apple with 70-75% degree of esterification was
purchased from Sigma-Aldrich Company (St. Louis, USA). RP
and ascorbyl palmitate (AP) were also purchased from Sigma-
Aldrich Company (St. Louis, USA). All other chemicals were of
analytical or high performance liquid chromatography (HPLC)
grade.

Preparation of transdermal formulations of RP

Test formulations of RP shown in Table 1 were prepared
by completely dissolving the ingredients in 1 ml of co-solvent
(ethanol:water=2:1). The formulations were stored at ambient
conditions prior to use.

Preparation of the skin

Male Wistar rats (Hanlim, Kyungkido, Korea) were used in
this investigation. The hairs of the rat dorsal skin surfaces
were carefully removed with an animal hair clipper and razors
without damaging the skin tissue. Skin samples were then ex-
cised after expiration under deep surgical anesthesia and the
subcutaneous lipid was subsequently removed. Prepared skin
samples were rinsed with PBS and stored at -70°C until the
skin permeation and distribution experiment.

In vitro skin permeation study using Franz diffusion cell

Franz diffusion cells (Model FCDV-15, Labfine Instruments,
Anyang, Korea) consisted of a donor compartment having an
effective diffusional area of 0.636 cm² and receiver compart-
ment with 5.0 ml volume capacity. The skins were sandwiched
between two compartments of the Franz diffusion cell. The
epidermal side of the skins was exposed to the donor com-
partment. Glycerin containing 80% ethanol was used as a re-
ceiver phase to ensure the sink condition. The receiver com-
partment was kept at 37 ± 0.5°C under magnetic stirring. The
mounted skins were equilibrated for 30 min, and then the air
bubbles were removed. A 1 ml of sample of each formulation
was placed on the skin surface and the donor compartment
was covered with a glass cap to prevent evaporation of the
vehicle. Samples (100 μl) of the receiver phase were withdraw-
at predetermined time intervals for HPLC determination of RP.

In vitro skin distribution of RP

At the end of the permeation experiment (24 hours), the
surface of the skin tissue was thoroughly washed 3 times with
PBS and gently dried with KimWipes (Yuhan-Kimberly, Gunpo,
Korea) to remove the drug associated with the skin surface.
The amount of RP in the SC was evaluated after tape-stripping
20 times using Scotch Tape (3M, St.Paul, MN, USA). The RP
was extracted from the tape by immersing it in 2 ml of metha-
nol, vortexing for 10 min and sonication for 30 min. The epider-
mis was then carefully removed from the dermis by immersing the SC-free
skin in distilled water maintained at 60°C for 1 min. The epider-
mis was then carefully removed from the dermis using forceps.
Each skin layer was cut into small pieces, immersed in metha-
nol 2 ml for extracting of RP and homogenized by Ultra-Turrax
homogenizer (IKA, Stafun, Germany) for 10 min. The resulting
mixtures were filtrated using 0.45 μm PVDF membranes and
amounts of RP were assayed by HPLC.

HPLC analysis of RP

Levels of RP were measured by HPLC. The HPLC system
consisted of a Waters 2695 Saprators Module (Alliance, Wa-
ters, Millford, MA, USA), Waters 2487 dual absorbance detect-
or (Alliance, Waters, Millford, MA, USA) and Hypersil Gold C18
column (5 μm, 4.6×250 mm). Methanol (100%) was used as a
mobile phase with a flow rate of 1.5 ml/min and the mobile
phase was monitored at 320 nm. The limit of detection of RP
was measured to be 0.052 μg/ml.

Statistical analysis

All reported data are mean ± standard deviation (n=4). Sta-
tistical significance was checked by one-way ANOVA and p<
0.05 was considered to be significantly different unless other-
wise indicated.

RESULTS

Effect of drug concentrations on skin distribution of RP

The effect of RP concentration in vehicles was first exam-

Table 1. Compositions of retinyl palmitate formulations

| Ingredients        | F1  | F2  | F3  | F4  | F5  | F6  | F7  | F8  |
|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Retinyl palmitate  | 1   | 2   | 3   | 3   | 3   | 3   | 3   | 3   |
| Pectin             | -   | -   | -   | 0.3 | 0.5 | 1   | -   | 0.5 |
| Ascorbyl palmitate | -   | -   | -   | -   | -   | -   | 0.1 | 0.1 |

All ingredients were solubilized in 1 ml of ethanol solution (EtOH:water=2:1). All quantities are given in mg.
ined in the absence of any stabilizers. As Figure 1 shows, the
RP deposition in SC and epidermis layers increased signifi-
cantly with increasing concentrations of RP indicating concen-
tration dependent RP penetration and deposition. However,
the amount of RP deposited in the dermis did not statistically
differ between the formulations. The amount of RP deposited
in epidermis was the greatest compared to those distributed in
SC and dermis. RP was not observed in the receiver compart-
ment during the course of the experiment possibly implying no
RP permeation across the skin layers.

Effect of pectin on skin distribution of RP
Pectin (0.3-1 mg) as an anti-oxidative stabilizer was added
to RP solution (3 mg/ml in ethanol solution, EtOH:water=2:1)
to explore the effect of pectin on the skin penetration and
thereby deposition of RP. At the concentrations of pectin test-
ed RP deposition behaviors were not considerably changed in
SC and dermis layers compared to that observed with the for-
mulations devoid of pectin (Fig. 2). However, the formulations
containing 0.5 and 1 mg of pectin (F4 and F5) demonstrated a
significantly increased RP distribution in the epidermis while
0.3 mg of pectin did not cause noticeably changes in the RP
deposition. The increased deposition of RP in the epidermis
might be due largely to the stabilization effect of pectin. To
further confirm this, the study on the effect of combined use
of pectin and well known anti-oxidant, ascorbyl palmitate (AP)
upon the skin deposition of RP was conducted with the for-
mulations listed in Table 1.

Effects of combined use of anti-oxidants on skin distribution
of RP
To confirm the stabilizing effect on RP distribution in the
skin AP (0.1 mg/ml) was added to RP formulations with and
without pectin. As illustrated in Fig. 3, AP (0.1 mg/ml) alone
did not appreciably change the skin deposition profiles of RP
exhibited by pectin (0.5 mg/ml) alone. However, the combined
use of AP (0.1 mg/ml) and pectin (0.5 mg/ml) obviously in-
creased the amount of RP deposited in the epidermis. How-
ever, no substantial increase in the deposition of RP in SC and
dermis was observed.

Time-dependent distribution of RP
Skin distribution profiles of RP formulated with pectin and
AP (F8) were observed as a function of time at 8, 16 and 24 h.
The depositions of RP in F3 formulation devoid of any stabiliz-
ers were slightly and greatly decreased in SC and epidermis,
respectively as time elapsed (Fig. 4). In contrast, the deposi-
tion amounts of RP in F8 formulation were slightly and greatly
increased in SC and epidermis, respectively as a function of
time. The RP distribution in SC was negligible measured at 8
h of the experiment but slightly increased as measured at 16 and 24 h (Fig. 4).

DISCUSSION

The skin is known as a strong barrier for the percutaneous absorption of drugs, in which the SC plays a main role in the prevention of the penetration of the drug (Scheuplein and Blank, 1971). When the drug is accumulated in the epidermal layer after penetrating the SC layer, it can be expected to constantly be delivered to the dermis which is the site of action of RP. Thus, the RP deposited in the epidermis would gradually increase the RP distribution in dermis. This subsequent accumulation can be explained by Fick’s first law of diffusion (Higuchi, 1960; Moser et al., 2001). Based on the law, drug permeation through SC can be improved by increasing drug concentration in the vehicles and therefore this might possibly support the increased RP permeation and deposition when increasing RP concentration in the vehicle. No drug was detected in the receiver phase although it is not a target site of RP. This might be attributed to the low skin permeability of retinoids (Teixeira et al., 2010) or the instability of RP under the experimental conditions employed.

Pectin at three different concentrations was added to RP solution to determine if there was concentration-dependent stabilization effect on the skin distribution behaviors of RP. The RP formulations containing more than 0.5 mg/ml of pectin showed the increased skin accumulation of RP compared to the formulation without pectin. Pectin showed the best in vitro stabilizing effect on RP among polysaccharides examined in our previous work (Ro et al., 2013). This stabilizing effect of pectin could prevent the degradation of RP in the donor compartment providing more RP available for permeation (data not shown). For this reason it seems that RP penetration into the skin tissue especially epidermis might largely be affected by the amount of pectin incorporated. However, the skin deposition of RP was not considerably improved with increasing concentration of pectin, especially at higher pectin concentrations. This result may stem from that high viscosity caused by higher amount of pectin prevented RP in the vehicles from free contact with the skin tissues. In case of SC, enhanced deposition of RP with a statistically significant difference was not observed in contrast to epidermis. This was maybe because SC is very thin tissue compared to epidermis, the thickest tissue in the skin, and thereby it was not feasible to achieve improved deposition of RP in SC with a statistically significant difference.

AP is synthetically-derived ester of ascorbic acid and widely used as an anti-oxidant. Additional stabilizer against the oxidation of RP was added to RP formulations containing pectin to investigate whether further stabilization of RP can alter the deposition profiles of RP in the skin layers. AP was selected due to its solubility in the vehicle used. It was clearly found that skin distribution of RP could be further enhanced by further increasing the stability of RP by AP due to its strong anti-oxidative effect (Cort, 1974; Hráš et al., 2000). For SC, enhanced deposition of RP with a statistically significant difference was not observed for the same reason described above.

In the skin distribution profiles as a function of time, the formulation containing RP alone (F3) seemed to be rapidly diffused to the skin tissues as measured at 8 h, but the amount of RP accumulated decreased gradually as time passed. The rapid penetration and deposition of RP in F3 formulation measured at 8 h might be probably due to lower viscosity of the formulation compared to F8 formulation containing pectin. The gradual or significant decrease in the accumulation of RP observed with F3 formulation could be the instability of RP limiting the amount of RP available for skin delivery. In the case of F8 formulation, it might be expected that the oxidative degradation of RP could be minimized because of the stabilization of RP by pectin and AP. This therefore caused the increased transdermal delivery of RP leading to more than three-fold greater accumulation of RP in the epidermis at 24 h, compared to F3 formulation.

In conclusion, these results clearly demonstrate that the skin deposition properties of RP can be improved by stabilizing RP with pectin and AP without using permeation enhancers frequently employed when increasing the delivery of drugs across the skin. Therefore, it can strongly be suggested that pectin may be used in the formulation design of RP to develop efficient pharmaceutical and cosmetic products.

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