S-methyl-L-methionine (SMM), also known as vitamin U, is a functional biomolecule having cationic structure with α-amino acid end group (Fig. 1). Natural sources of SMM are mainly raw cabbage and green vegetables such as broccoli and celery. Its molecular weight is 164.25 g/mol with a log p-value of -3.3, which indicates that it is very hydrophilic (Patel and Prajapati, 2012).

Diverse pharmacological efficacy of SMM has been reported. Oral administration of SMM reduced the liver damage induced by valproic acid in rats (Sokmen et al., 2012). In experimental nephrotic hyperlipidemia, SMM ameliorated cholesterol and phospholipid levels in blood (Seri et al., 1979). Moreover, SMM showed protective effect not only on rat gastro-intestinal mucosa (Ichikawa et al., 2009) but also on oesophago-gastric ulcers in pig studies (Kopinski et al., 2007).

Studies on the effect of topical skin administration of SMM have begun recently. The topical administration of SMM enhanced the repair of skin damage by activation of fibroblasts, thereby showing a wound healing effect (Kim et al., 2010). SMM also showed a photoprotective effect by reducing UV-induced skin damage (Kim et al., 2015). Based on these results, SMM was developed as a wound healing agent and skin care cosmetic product. However, there has been no systematic study reported up to date on the skin permeation/deposition of SMM even though the skin permeation of SMM is expected to be low due to its high hydrophilicity. Thus, this study has been initiated with the assumption that the use of skin permeation enhancers could improve the topical delivery of SMM.

Key Words: S-methyl-L-methionine, Vitamin U, Skin permeation, Deposition, Enhancers

INTRODUCTION

S-methyl-L-methionine (SMM), also known as vitamin U, is a functional biomolecule having cationic structure with α-amino acid end group (Fig. 1). Natural sources of SMM are mainly raw cabbage and green vegetables such as broccoli and celery. Its molecular weight is 164.25 g/mol with a log p-value of -3.3, which indicates that it is very hydrophilic (Patel and Prajapati, 2012).

Diverse pharmacological efficacy of SMM has been reported. Oral administration of SMM reduced the liver damage induced by valproic acid in rats (Sokmen et al., 2012). In experimental nephrotic hyperlipidemia, SMM ameliorated cholesterol and phospholipid levels in blood (Seri et al., 1979). Moreover, SMM showed protective effect not only on rat gastro-intestinal mucosa (Ichikawa et al., 2009) but also on oesophago-gastric ulcers in pig studies (Kopinski et al., 2007).

Studies on the effect of topical skin administration of SMM have begun recently. The topical administration of SMM enhanced the repair of skin damage by activation of fibroblasts, thereby showing a wound healing effect (Kim et al., 2010). SMM also showed a photoprotective effect by reducing UV-induced skin damage (Kim et al., 2015). Based on these results, SMM was developed as a wound healing agent and skin care cosmetic product. However, there has been no systematic study reported up to date on the skin permeation/deposition of SMM even though the skin permeation of SMM is expected to be low due to its high hydrophilicity. Thus, this study has been initiated with the assumption that the use of skin permeation enhancers could improve the topical delivery of SMM.

Abstract

S-methyl-L-methionine (SMM), also known as vitamin U, is commercially available as skin care cosmetic products for its wound healing and photoprotective effects. However, the low skin permeation expected of SMM due to its hydrophilic nature with a log p-value of -3.3, has not been thoroughly addressed. The purpose of this study thus was to evaluate the effect of skin permeation enhancers on the skin permeation/deposition of SMM. Among the enhancers tested for the in vitro skin permeation and deposition of SMM, oleic acid showed the most significant enhancing effect. Moreover, the combination of oleic acid and ethanol further enhanced in vitro permeation and deposition of SMM through hairless mouse skin. Furthermore, the combination of oleic acid and ethanol significantly increased the in vivo deposition of SMM in the epidermis/dermis for 12 hr, which was high enough to exert a therapeutic effect. Therefore, based on the in vitro and in vivo studies, the combination of oleic acid and ethanol was shown to be effective in improving the topical skin delivery of SMM, which may be applied in the cosmetic production process for SMM.

Key Words: S-methyl-L-methionine, Vitamin U, Skin permeation, Deposition, Enhancers
enhancer could improve the skin permeation and/or deposition of SMM, thereby further increasing its therapeutic efficacy. Various skin permeation enhancers with different mechanisms of action have been reported (Rachakonda et al., 2008). However, only a few could significantly enhance the skin permeation of hydrophilic compounds (Karande et al., 2004), and skin irritation has always been a critical issue (Lashmar et al., 1989). Thus, the objective of this study was to optimize and evaluate the skin permeation enhancer on the in vitro skin permeation and deposition of SMM in hairless mouse model. Also, the synergistic effect of skin permeation enhancers on in vitro and in vivo skin deposition of SMM was systematically investigated.

MATERIALS AND METHODS

Materials

SMM was purchased from Sigma Chemical Co (St. Louis, MO, USA). Propylene glycol (PG) was purchased from Samchun Chemical Co., Ltd (Pyeongtaek, Korea). Isopropyl myristate (IPM), oleic acid, and limonene were purchased from Sigma Chemical Co. HPLC grade methanol, ethanol and acetonitrile were purchased from Thermo Fisher Scientific Co (Pittsburgh, PA, USA).

Animals

For in vitro and in vivo evaluation, male hairless mice (18-20 g) were obtained from Orient Bio Inc (Sungnam, Korea). They had free access to water and food before the studies. Experimental protocols for the animals (SNU-111007-4-2) used in this study were reviewed by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University (Seoul, Korea) and were in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication Number 85-23, revised 1985).

In vitro skin permeation and deposition studies

Evaluation of in vitro permeation and deposition of SMM through hairless mouse skin was carried out by using Keshary-Chien diffusion cells at 32°C, which have 1.77 cm² of the surface area for diffusion. After sacrificing the hairless mice by cervical dislocation, the dorsal skin was cut to about 3 cm x 3 cm size and the subcutaneous fat was removed. Then, they were fixed between the donor and receptor cells, laying the stratum corneum toward the donor cells. The donor cells contained 2% (w/v) or 5% (w/v) SMM in the 50:50 (v/v) mixture (1.0 mL) of PG and double distilled water (DDW) with or without permeation enhancers, and were covered with paraffin to prevent evaporation. The receptor cells were filled with DDW (13.0 mL) and continuously stirred by magnetic bar. After applying SMM solution on the donor cells, 0.5 mL of the receptor solution was collected at 3, 6, 9, and 12 hr and added immediately with an equal volume of fresh media. SMM in the receptor solutions was analyzed using LC-MS/MS.

For in vitro skin deposition studies, the skin was removed from the diffusion cells at 6 and 12 hr after applying SMM solution and washed out with methanol. In order to determine the amount of SMM in stratum corneum by tape stripping method, cellophane adhesive tape (CuDerm Coperation, Dallas, TX, USA) was applied three times on the stratum corneum of the skin. Then, the tapes of each time samples were separately collected into the 15 mL tube and added with mobile phase (3.0 mL). SMM was extracted from the tapes by shaking the tube for 3 hr, followed by centrifugation for 5.0 min at 16,100 g. The skin samples after removing the stratum corneum (i.e., epidermis and dermis) were chopped and collected into the 15 mL tube. After adding mobile phase (3.0 mL), they were homogenized using ULTRA-TURAX® T25basic (IKA, Staufen, Germany), followed by centrifugation for 1.5 min at 3,660 g. The amount of SMM in the stratum corneum and epidermis/dermis at 6 and 12 hr was analyzed using LC-MS/MS.

In vivo skin deposition study

Hairless mice were lightly anesthetized with ether and each mouse was fixed laying the dorsal skin upward. A specially designed cylinder-type chamber which has a diffusion area of 2.14 cm² was put on the dorsal skin of mice and fixed with surgical glue (Vet bond®, 3M Co., St. Paul, MN, USA) (Valiveti et al., 2004; Jung et al., 2013). Aliquot of PG solution containing 2% (w/v) of SMM with or without enhancer [i.e., 2% (w/v) oleic acid with or without 10% (w/v) ethanol] was applied into the chamber for topical administration of SMM at 80 mg/kg dose. The hairless mice were sacrificed by cervical dislocation at 6 and 12 hr, and then the skin samples were prepared as described in in vitro skin deposition study to determine the amount of SMM.

LC-MS/MS analysis of SMM

SMM was analyzed by using LC-MS/MS following the method in the literature with slight modification (Scherb et al., 2009). Samples were transferred to the mass vials and a 3 μL aliquot was injected into Synergi Max-RP column (75 mm×4.6 mm, 4 μm; Phenomenex, Torrance, CA, USA). The mobile phase was 73% acetonitrile and 27% DDW containing 0.2% formic acid at the flow rate of 0.5 mL/min. The column eluents were detected with an Agilent LC-MS/MS system equipped with an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA) and Agilent Technologies 6430 Triple Quad LC-MS system. Optimization was conducted in the multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI). The gas temperature, gas flow, nebulizer pressure, and capillary voltage were 300°C, 11 L/min, 15 psi, and 4500 V, respectively. The m/z value of precursor to product ion, fragment voltage, collision energy, and cell accelerator voltage were 163.9 to 102.0 (Fig. S1), 73 V, 7eV, and 1 V, respectively. The data were processed using the MassHunter Workstation Software Quantitative Analysis (vB.05.00; Agilent Technologies). The retention time of SMM was 1.1 minutes (Fig. S2a). The mean correlation coefficient (R2) for the calibration curve was over 0.999 (Fig. S2b). The lower limit of quantification (LLOQ) of SMM was 20 ng/mL. Precision and accuracy values were within acceptable ranges (Table S1).

Statistical analysis

All experiments were carried out at least three times (n≥ 3). A p-value less than 0.05 was considered to be statistically significant using one-way ANOVA. Data were shown as mean ± standard deviation.
**RESULTS**

Effect of SMM concentration on *in vitro* skin permeation and deposition

The *in vitro* skin permeation study showed that the amount of SMM in the receptor solution after 12 hr of applying up to 20% (w/v) of SMM was below the detection limit (data not shown). However, the skin deposition of SMM after applying 2% (w/v) or 5% (w/v) of SMM, which were selected based on the efficacy studies in the literature (Kim et al., 2010, 2015), was detectable by LC-MS/MS analysis. As shown in Fig. 2, application of 5% (w/v) SMM resulted in higher deposition at both 6 hr and 12 hr than those of 2% (w/v). It is also interesting to note that the deposition of SMM in the epidermis/dermis was always higher than that of the stratum corneum, regardless of application time and SMM concentration. Since application of 2% (w/v) SMM was high enough to determine its skin deposition, further studies to investigate the effect of permeation enhancer was conducted at this concentration.

**Effect of absorption enhancers (5%, w/v) on the *in vitro* skin permeation and deposition of SMM**

To investigate the effect of enhancers on the *in vitro* skin permeation and deposition of SMM (2%, w/v), three different enhancers were added at 5% (w/v) in the donor solution. Fig. 3A shows the *in vitro* skin permeation profiles of SMM, and its permeation parameters are summarized in Table 1. Skin permeation of SMM without enhancer was negligible, and was below the detection limit. However, the addition of enhancers increased the flux of SMM compared with the control group (without enhancer). The most effective enhancer among tested was oleic acid, which significantly increased the flux of SMM up to 7.98 ± 4.43 μg/hr/cm². Fig. 3B shows the effect of enhancer on the *in vitro* skin deposition of SMM at 6 hr and 12 hr. Oleic acid significantly increased the amount of SMM in both the stratum corneum and epidermis/dermis at 6 hr and 12 hr, which is consistent with the results of the *in vitro* skin permeation study (Fig. 3A, Table 1).

**Table 1. Effect of absorption enhancers (5%, w/v) on the *in vitro* skin permeation parameters of SMM dissolved in the 50:50 (v/v) mixture of PG and DDW at 2% (w/v)**

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Flux (μg/hr/cm²)</th>
<th>Permeability coefficient (×10⁻⁴ cm/hr)</th>
<th>Lag time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without enhancers</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>0.35 ± 0.36</td>
<td>0.18 ± 0.18</td>
<td>3.46 ± 0.66</td>
</tr>
<tr>
<td>Limonene</td>
<td>1.38 ± 0.82</td>
<td>0.69 ± 0.41</td>
<td>1.51 ± 0.43</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>7.98 ± 4.43*#</td>
<td>3.99 ± 2.21*#</td>
<td>4.11 ± 0.25</td>
</tr>
</tbody>
</table>

ND: below the detection limit.
*Significantly different from the IPM group (p<0.05).
**Significantly different from the Limonene group (p<0.05).

**Effect of oleic acid concentration on *in vitro* skin permeation and deposition of SMM**

Fig. 4A shows the effect of oleic acid concentration on the *in vitro* skin permeation of SMM, and Table 2 summarizes the permeation parameters. Although oleic acid increased the skin permeation of SMM in a dose-dependent manner, significant increase of the SMM flux was observed in the 5% oleic acid group only (Table 2). Moreover, skin deposition study also consistently show that the amount of SMM in the stratum corneum and epidermis/dermis significantly increased with the addition of 5% oleic acid only (Fig. 4B). The amount of SMM in the epidermis/dermis at 12 hr was 9.43 times higher in 5% oleic acid group compared with the control group (without enhancer). Although oleic acid was the most effective skin permeation enhancer for SMM among tested, it is known to cause skin irritation at higher than 5% concentration (Songkro,
Thus, further study on the synergistic skin permeation-enhancing effect of ethanol and oleic acid was investigated to minimize the use of oleic acid.

Synergistic effect of oleic acid and ethanol on in vitro permeation and deposition of SMM

In vitro skin permeation profiles of SMM when 10% ethanol was added alone or in combination with oleic acid are shown in Fig. 5A. Synergistic effect of ethanol and oleic acid on the permeation parameters is summarized in Table 3. Addition of 10% ethanol (without oleic acid) showed a permeation-enhancing effect, and increased the flux of SMM up to 0.27 μg/hr/cm², which is comparable to the effect of 2% oleic acid (Table 2). Moreover, when 10% ethanol was added with 1% oleic acid, the flux of SMM (7.11 μg/hr/cm²) was comparably enhanced to that with 5% oleic acid (7.98 μg/hr/cm², Table 1).

When 2% oleic acid was added with 10% ethanol, the flux of SMM synergistically increased up to 19.83 μg/hr/cm² (Table 2). The lag time also showed decreasing tendency when ethanol and oleic acid were added in combination. The lag time of permeation decreased to 3.22 hr when 2% oleic acid was added with 10% ethanol (Table 3), while that of 2% oleic acid alone was 4.29 hr (Table 2). Fig. 5B shows that the in vitro skin deposition of SMM synergistically increased when oleic acid was added with 10% ethanol. The amount of SMM in epidermis/dermis at 12 hr increased 12.3 time when 2% oleic acid and 10% ethanol was added in combination, compared to the control group (without enhancer).

In vivo skin deposition of SMM in hairless mice

The effect of permeation enhancer on the in vivo skin deposition of SMM was consistent with that of the in vitro result, as shown in Fig. 6. The addition of 2% oleic acid increased
**Table 3.** Synergistic effect of oleic acid and ethanol on the in vitro skin permeation parameters of SMM dissolved in the 50:50 (v/v) mixture of PG and DDW at 2% (w/v).

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Flux (µg/hr/cm²)</th>
<th>Permeability coefficient (×10⁻⁴ cm/hr)</th>
<th>Lag time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without enhancers</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10% ethanol</td>
<td>0.27 ± 0.34</td>
<td>0.14 ± 0.17</td>
<td>ND</td>
</tr>
<tr>
<td>1% oleic acid</td>
<td>7.11 ± 0.84*</td>
<td>3.56 ± 0.42*</td>
<td>3.85 ± 0.29</td>
</tr>
<tr>
<td>10% ethanol+</td>
<td>19.83 ± 3.33**</td>
<td>9.91 ± 1.67**</td>
<td>3.22 ± 0.07</td>
</tr>
<tr>
<td>2% oleic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% ethanol+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% oleic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% oleic acid+10% ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND: below the detection limit.
*Significantly different from the 1% oleic acid group (p<0.05).
**Significantly different from the 10% ethanol+1% oleic acid group (p<0.05).

![Graph showing synergistic effect of oleic acid and ethanol on in vitro skin permeation of SMM.](https://doi.org/10.4062/biomolther.2016.254)

**Fig. 6.** Effect of oleic acid and ethanol on the in vivo skin deposition of SMM at 6 hr and 12 hr in the stratum corneum (SC) and the epidermis/dermis of hairless mouse skin after applying 2% (w/v) of SMM in the 50:50 (v/v) mixture of PG and DDW (Mean ± SD, n=3).

- *p<0.05, ***p<0.001.

The deposition of SMM in the epidermis/dermis at 6 hr and 12 hr, compared to the control (without enhancer). Moreover, 2% oleic acid and 10% ethanol synergistically increased the deposition of SMM up to 4.65 µg/cm² at 12 hr in epidermis/dermis, which was 4.2 times higher than that of the control.

**DISCUSSION**

The target site of SMM for wound healing and photoprotective effect is the epidermis/dermis of the skin which includes the fibroblast and keratinocyte (Barry, 2001; Benson, 2005). However, the stratum corneum on the outermost layer of the skin works as an absorption barrier, and thus would be the main hurdle for the topical skin delivery of SMM. Since intercellular micro-route of stratum corneum is lipophilic in nature, the molecule with optimum lipophilicity is a prerequisite for permeation. However, SMM is a hydrophilic compound with a log p-value of -3.3, which leads to the expectation that the skin permeation of SMM would be poor (Patel and Prajapati, 2012).

Since topical skin delivery of SMM has not been previously reported, the goal of this study was therefore to first of all evaluate the skin permeation and deposition of SMM, after which to investigate the effect of enhancers. Fortunately, the skin deposition of SMM in epidermis/dermis was detectable by LC-MS/MS analysis (Fig. 2), although the in vitro skin permeation without enhancer was not (Table 1). However, the amount of SMM from the in vivo skin deposition study was less than that from the in vitro study (Fig. 5B vs. Fig. 6), which also should be considered when conducting in vitro study to estimate in vivo skin deposition.

Due to the difficulties in obtaining human skin and its variability of lipid content, animal models, particularly hairless mouse whose hair follicles are underdeveloped and less dense (Lauer et al., 1995), have been generally used as an alternative to the human skin (Bond and Barry, 1988). In many studies, the correlation of permeation characteristics between hairless mouse and human skins has been reported for many drugs, which is due to the similarity of skin lipid composition between two skins (Durrheim et al., 1980; Jung and Maitlach, 2015; Jung et al., 2015). It is well known that the major skin permeation route is transepidermal pathway, and transappendageal pathway (including hair follicles and sweat gland) contributes less than 0.1% of the total permeation amount (Bond and Barry, 1988). Moreover, it was reported that in vivo absorption of ionic solutions through hairy skin and hairless skin was not significantly different (Wahlberg, 1968). Based on these previous literatures, we believe that hairless mouse is a suitable model, and hair follicles in hairless mouse do not affect the in vitro and in vivo skin permeation of SMM.

In order to further increase the skin permeation and deposition in topical delivery, formulation with skin permeation enhancers is a simple and useful method (Songkro, 2009; Marwah et al., 2016). Results of this study showed that oleic acid, alone and in combination with ethanol, is a useful enhancer for SMM among tested, and significantly increases both in vitro skin permeation and deposition of SMM. Oleic acid is one of the most potent skin permeation enhancers and has a different mechanism on enhancing skin permeability from its cis unsaturated double bonds structure (Trommer and Neubert, 2006). Due to its structure, oleic acid is known to increase the fluidity of the stratum corneum and form separate phases within the bilayer lipids in stratum corneum, thereby forming a permeable interfacial defect to increase permeation of hydrophilic molecules through the skin (Koyama et al., 1994; Trommer and Neubert, 2006; Songkro, 2009). However, addition of more than 5% oleic acid causes skin irritation (Sintov et al., 1998; Songkro, 2009), and thus less than 2% is recommended for topical skin delivery formulations (Sintov et al., 1998). Diverse skin permeation-enhancing mechanisms of ethanol have been reported, which include lipid extraction effect (Lachenmeier, 2008). High concentration of ethanol also could cause skin irritation, but up to 10% ethanol in formulation is commonly used without adverse effects (Lachenmeier, 2008). Moreover, many studies already reported that the combination of oleic acid and ethanol synergistically enhance the skin permeation and deposition in skin delivery (Koyama et al., 1994; Kim and Chien, 1995; Jung et al., 2013). Consistent with the previous reports, 1% oleic acid in combination with 10% ethanol showed comparable in vitro skin permeation of SMM to that of 5% oleic acid (7.11 µg/hr/cm² vs. 7.58 µg/hr/cm², Table 1, 2). Moreover, 2% oleic acid in combination with 10% ethanol further increased the flux of SMM up to 19.83 µg/hr/cm² (Table 2). It is also notable that the in vitro skin deposition of SMM synergistically increased by combining oleic
achieved when 2% oleic acid in combination with 10% ethanol resulted in 4.65 μg/cm²: SMM at 12 hr in epidermis/dermis (Fig. 6).

The concentration of SMM in its target epidermis/dermis is closely related to its efficacy for skin care cosmetic products. In the in vivo skin deposition study, the amount of SMM in epidermis/dermis at 12 hr without permeation enhancer (1.10 μg/cm², Fig. 6) was converted to its SMM concentration, assuming that the thickness of epidermis/dermis of hairless mouse is about 300 μm (Calabro et al., 2011) and SMM is homogeneously distributed. The calculated concentration of SMM in the 2.14 cm² surface area of epidermis/dermis was about 223 μM. The skin permeability of human skin is known to be at least two times lower than that of hairless mouse skin (Jung et al., 2015; Seo et al., 2016), and SMM showed wound healing and photoprotective effect in human dermal fibroblast at higher than 100 μM concentration (Kim et al., 2010, 2015). Thus, it was necessary to further increase the skin deposition of SMM in epidermis/dermis. The amount of SMM in epidermis/dermis at 12 hr by the addition of 2% oleic acid and 10% ethanol was 4.65 μg/cm², which is equivalent to about 943 μM.

Thus, it can be concluded that the therapeutically significant concentration of in vivo skin deposition of SMM can be achieved when 2% oleic acid in combination with 10% ethanol was used as permeation enhancer. Further studies are underway in this laboratory to develop gel-type topical formulations of SMM.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Korea (HN14C0084).

REFERENCES


