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Effect of Vehicles and Enhancers on the *in vitro* Skin Penetration of Aspalatone and Its Enzymatic Degradation Across Rat Skins

Hye Sun Gwak and In Koo Chun

Laboratory of Pharmaceutics, College of Pharmacy, Dongduk Women's University, Seoul 136-714, Korea

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The feasibility of skin penetration was studied for aspalatone (AM, acetylsalicylic acid maltol ester), a novel antithrombotic agent. In this study, hairless mouse dorsal skins were used as a model to select composition of vehicle and AM. Based on measurements of solubility and partition coefficient, the concentration of PG that showed the highest flux for AM across the hairless mouse skin was found to be 40%. The cumulative amount permeated at 48 h, however, appear inadequate, even when the PG concentration was employed. To identify a suitable absorption enhancer and its optimal concentration for AM, a number of absorption enhancers and a variety of concentration were screened for the increase in transdermal flux of AM. Amongst these, linoleic acid (LOA) at the concentration of 5% was found to have the largest enhancement factor (i.e., 132). However, a further increase in AM flux was not found in the fatty acid concentration greater than 5%, indicating the enhancement effect is in a bell-shaped curve. In a study of the effect of AM concentration on the permeation, there was no difference in the permeation rate between 0.5 and 1% for AM, below its saturated concentration. At the donor concentration of 2%, over the saturated condition, the flux of AM was markedly increased. A considerable degradation of AM was found during permeation studies, and the extent was correlated with protein concentrations in the epidermal and serosal extracts, and skin homogenates. In rat dorsal skins, the protein concentration decreased in the rank order of skin homogenate > serosal extract > epidermal extract. Estimated first order degradation rate constants were 6.15 ± 0.14, 0.57 ± 0.02 and 0.011 ± 0.004 h^{-1} for skin homogenate, serosal extract and epidermal extract, respectively. Therefore, it appeared that AM was hydrolyzed to some extent into salicylmaltol by esterases in the dermal and subcutaneous tissues of skin. Taken together, our data indicated that transdermal delivery of AM is feasible when the combination of PG and LOA is used as a vehicle. However, since AM is not metabolically stable, acceptable degradation inhibitors may be necessary to fully realize the transdermal delivery of the drug.

Key words: Aspalatone, Percutaneous absorption, Vehicles, Penetration enhancers, Degradation

INTRODUCTION

Acetylsalicylic acid (ASA) is known to exert its antithrombotic effect by its inhibitory effect on platelet cyclooxygenase by the irreversible acetylation, and now being widely used for the treatment and prevention of cardiovascular disease (Roth and Majerus, 1975; Loll *et al.*, 1995). ASA, however, has been associated with many gastrointestinal side effects due to the loss of cytoprotective effects of PGE_2 on the gastric mucosa (Awtry and Loscalzo, 2000). Furthermore, ASA inhibits not only the production of proaggregatory thromboxane A_2 (TXA_2) in platelets but also the production of antiaggregatory prostacyclin (PGI_2) in vessel walls, known as aspirin dilemma (Patrignani *et al.*, 1982; Patrono, 1994). To overcome these problems of ASA, aspalatone (AM, acetylsalicylic acid maltol ester, [3-(2-methyl-4-pyronyl)]-2-acetoxybenzoate) was synthesized by esterification of ASA and maltol, an active antioxidant component in Korean red

Correspondence to: In Koo Chun, College of Pharmacy, Dongduk Womens University, Seoul 136-714, Korea
E-mail: ikchun@dongduk.ac.kr

ginseng (Han *et al.*, 1994). Some studies have shown that AM had a potential antithrombotic activity with a low ulcerogenicity (Suh *et al.*, 1996).

Even though AM was considered as a novel antithrombotic agent, only a small amount of the drug was found intact in an *in vitro* study involving an incubation of rabbit gastrointestinal tissues (Chun and Gwak, 2001) with AM. As a result, flux of intact AM across gastrointestinal membrane is likely to be low, and, thus, oral administration of the drug may not be practical. Currently, no other route of administration is available for this drug. In this study, therefore, feasibility of transdermal delivery was studied for a potential route of administration of AM. Thus, we examined the permeation and enzymatic degradation of AM through excised rat dorsal skins, and screened for penetration enhancers for the improvement of the transdermal transport of AM in the *in vitro* model.

MATERIALS AND METHODS

Materials

AM and salicylmaltol (SM) were kindly provided by Bukwang Pharm. Ind. Co. Ltd (Seoul, Korea). ASA, salicylic acid (SA), oleic acid (OA), linoleic acid (LOA), lauric acid (LA), oleyl alcohol (OAl), sodium lauryl sulfate (SLS), and rabbit serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). Phospholipid (Lipoid S100) was obtained from Lipoid KG (Ludwigshafen, Germany). Acetonitrile and methanol used were of HPLC grade. Other reagents were of analytical grade.

Animals

Male rats (Sprague-Dawley strain) with an approximate weight of 300~350 g and male hairless mice aged 6~8 weeks were purchased from Seoul National University Hospital Animal Laboratory Service and Samtako Bio Korea Co., Ltd. (Osan, Korea), respectively.

HPLC Analysis of AM

Samples from permeation and degradation studies were analyzed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a pump (Perkin-Elmer Series 410, Norwalk, USA), a detector (Model LC 90, Perkin-Elmer, Norwalk, USA) and an integrator (Varian, Model 4290, Walnut Creek, USA). The eluent from a reversed HPLC column (Ultrasphere C8 column, Beckman Instruments Inc., Fullerton, USA) was monitored at 229 nm. The mobile phase was composed of 1/15 M phosphate buffer (pH 3.0), acetonitrile and methanol (65.5:32:2.5), and delivered at a flow rate of 1.0 ml/min. The injection volume was 20 μl. When it was necessary, a calibration curve was constructed to estimate concentration in *in vitro* studies. In the calibration study, peak area was used as assay parameter.

In vitro skin permeation studies

To determine the effects of various vehicles and enhancers on transdermal permeation of AM, saturated solutions were prepared by shaking an excess amount of AM in the pure solvents or cosolvents in the presence and the absence of enhancers at 37°C for 24 h. To study the drug permeation, rat skin with the full thickness was used. Upon the sacrifice by euthanasia with ether, the dorsal region was carefully shaved with electric razors, and then excised. Within 30 min after the removal of the subcutaneous fat layer with a scrubbing, the excised skin was mounted on a side-by-side permeation system (Valia-Chien Permeation System, Crown Bioscientific Inc., New Jersey, USA); the epidermal side was in contact with the receptor compartment. The surface area of the epidermis exposed to the solution was 0.64 cm^2. Receptor compartment cells were filled with 3.5 ml of 40% PEG 400 in saline, and the media were stirred during the experiment by a Teflon-coated magnetic bar at 600 rpm to keep them well mixed. Donor compartment cells were filled with 3.5 ml of saturated drug solutions in various pure solvents or cosolvents. Temperature of cells was maintained at 37°C using a heating circulator. At predetermined time intervals, 100 μl of receptor solutions were withdrawn from the receptor cells, and mixed with 900 μl of pH 2.2 phosphate buffer to terminate potential enzymatic degradation. Equivalent amount of 40% PEG 400 in saline was added to receptor cells in order to maintain a constant volume. The permeated amount was determined by HPLC assay for AM.

Preparation of skin extracts and homogenates

The rat skin preparation and the mounting of the excised rat skin was carried out similar to that described in the previous section. Each compartment was filled with saline, and stirred at 600 rpm for 6 h by a Teflon-coated magnetic bar to keep them well mixed. The epidermal and serosal extract solutions were collected from the donor and receptor cells, respectively. The skin extracts were used in subsequent studies without further preparation.

For skin homogenates, approximately 4 g of the excised and sliced skin from the rat was added to 19 ml of saline, and homogenized using Ultra-Turrax T25 for 1 h in an ice bath. The homogenate was centrifuged at 3700 rpm for 10 min, and then 1.8 ml of the supernatant was collected for subsequent studies.

Determination of protein concentration in skin extracts and homogenates

Protein concentrations in the epidermal, serosal extracts, and homogenates were measured using the method des-

cribed by Schosinsky *et al.* (1987). The protein concentration of the homogenate was diluted 100 times with saline. Protein determination for skin extracts was carried out without further dilution. Briefly, an aliquot (0.5 ml) of the sample was added to 2.0 ml of bromophenol blue solution, and absorbance at 610 nm was determined spectrophotometrically. The concentration of protein was calculated from a standard curve obtained using rabbit serum albumin.

Degradation studies using skin extracts and homogenates

For skin extracts, 200 μl of AM solution (1,000 μg/ml) was added to 1800 μl of the epidermal and serosal extracts. The mixture was gently shaken at 60 rpm in a shaking water bath set at 37°C. At predetermined time intervals, 100 μl of solutions were withdrawn, and mixed with 900 μl of pH 2.2 phosphate buffer to terminate further enzymatic degradation. The intact AM in the epidermal and serosal extracts was determined by HPLC. For skin homogenates, 200 μl of AM solution (1000 μg/ml) was added to 1800 μl of the homogenate (1,449 μg/ml), and then incubated at 37°C. At predetermined time intervals, 100 μl of solution was withdrawn, and mixed with 200 l of acetonitrile to remove protein residue. It was further diluted with 700 μl of pH 2.2 phosphate buffer, and centrifuged at 10,000 rpm for 5 min. The intact AM was determined by HPLC.

RESULTS AND DISCUSSION

Effect of PG-water cosolvents on the permeation of AM

Ostrenga *et al.* (1971) showed that the solubility and partition characteristics of fluocinolone and its ester could be modified with a varying ratio of water and PG. These investigators were found that the most effective formulations for the two steroids were where the solubility and partition coefficient lines intersect. As shown in Fig. 1, relative solubility and partition coefficient were found to intersect approximately at 40% PG. Based on the characteristics found in the steroids, the most appropriate concentration for the transdermal delivery of AM appeared to be based on the studies of the solubility and partition coefficient of AM (Gwak and Chun, 2000). Consistent with the expectation, transdermal flux study agreed with the selected concentration for AM (Fig. 2). The permeated amount through 48 hr using 40% PG was 15 and 2.5 times higher than those using 1/15 M phosphate buffer (pH 6.0) and pure PG, respectively. The cumulative amount permeated at 48 h, however, was too low, which was 4.13 μg/cm^2 even when using 40% PG. This low permeability appeared related to the presence of the stratum corneum, which is known to have an extremely

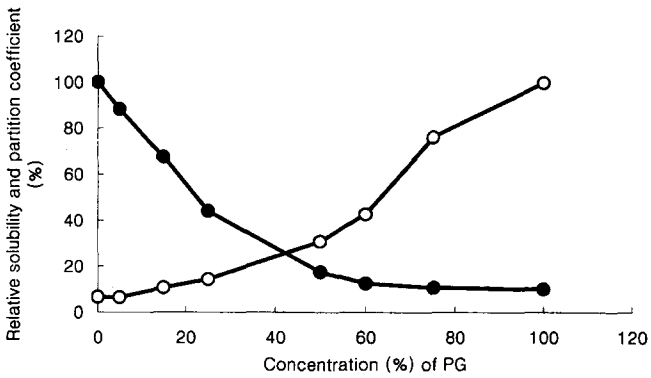

Fig. 1. Relation of solubility and Pc profile of AM in PG-water co-solvent system at 32°C. The oil phase used for Pc was isopropyl myristate. Each value represents the mean of three determinations. Key: blank circle, relative solubility; filled circle, relative partition coefficient.

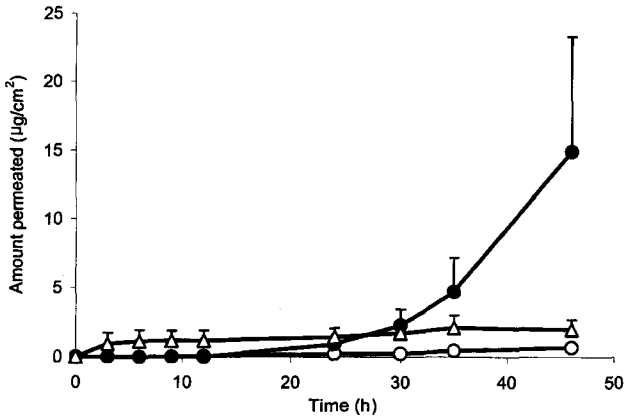

Fig. 2. Effect of PC-water co-solvent system on the permeation of AM across the excised rat skin. AM was added in a saturating condition in each vehicle composition. Each value represents the mean ± S.E. (n = 3). Key: blank circle, pH 6.0 buffer only; filled circle, 40% PG; blank triangle, PG only.

good barrier property against skin penetration. A number of strategies may be employed to overcome this barrier property. Increase in drug solubility in skin; dissoluteion skin lipids; alteration of the conformation or denaturing skin proteins; disruption of water structure in skin; and increase in membrane fluidity may be applic-able to alter the barrier function of the stratum corneum (Aungst *et al.*, 1986).

Effect of enhancers on the flux of AM

PG is known to solvate alpha keratin and to occupy hydrogen-bonding sites, and thus reducing drug/tissue binding. However, it does not appear to alter the lipid fluidity by influencing horny layer lipid structure (Barry, 1987). On the contrary, fatty acids or fatty alcohols are reported to reduce the skin resistance by disruption of

tightly packed lipid regions of stratum corneum, which would lead to an increase in penetration of solutes through the intercellular lipid matrix (Yamada *et al.*, 1987; Aungst *et al.*, 1990). Thus when used with PG, these enhancers such as fatty acids and fatty alcohols would synergistically increase the transdermal transport of solutes by affecting lipid region (Nomura *et al.*, 1990). Based on these reasoning, fatty acids such as OA, LOA and LA, and fatty alcohols like OAl were employed in this study as enhancers with lipophilic properties. Table I shows the permeation parameters when enhancers such as OA, LA, and OA with phospholipids using 40% PG as a vehicle. The concentration of AM used was 0.5%, which was saturated concentration in 40% PG. The enhancement factors by using OA, LA and OA with phospholipid were 2.6, 10.8 and 12.9, respectively; and the addition of phospholipid to OA did not increase the flux significantly. These observations appeared consistent with the proposed mechanisms of these enhancers (Yamada *et al.*, 1987; Aungst *et al.*, 1990; Nomura *et al.*, 1990)

Table II represents the flux and lag time of AM when using pure PG as a vehicle. The enhancers employed in this study were OA, LOA, LA, OAl and SLS. The enhancing effect of LA, and OAl was much less than that of unsaturated fatty acids. The enhancing effects by unsaturated fatty acids were dramatic, especially for LOA (i.e., the flux was 23.8 $\mu g/cm^2/h$). The mechanisms for the dramatic enhancing effect of unsaturated fatty acids and fatty alcohol were not directly studied for AM. However, the literature evidence suggested the number of double bonds correlated well with the enhancement effect for fatty acids (Aungst, 1986). For fatty alcohols, they may interact with phospholipids at the boundary lipid layer by hydrophobic interaction, but less than their acid analogues, which may lead to less membrane fluidization effect of alcohols than acids (Kitagawa *et al.*, 1985).

Levang *et al.* (1999) suggested that ASA dose required for the inhibition of platelet formation is 21 mg/day considering the pharmacological activity and oral bioavailability. In addition, the literature showed that the *in vitro* IC_{50} value of AM for collagen-induced platelet aggregation was comparable to that of ASA (Han *et al.*, 1994). Therefore, if a patch was employed in a size of 70 cm^2 and the composition of 5% LOA in PG (i.e., the flux was 23.8 $\mu g/cm^2/h$), the delivery amount for AM would be 40 mg/day. Thus, it would be feasible to reach the dosing rate for inhibition of platelet formation.

Anionic surfactants like SLS are known to disrupt protein structure, which also results in a loss of water binding capacity (Scheuplein and Ross, 1970). In addition, a study has shown that SLS increased the skin penetration of urea and pentanol 8300-fold and 7-fold, respectively (Cooper, 1982). Despite the proposed mechanisms for the enhancing effect of SLS (Hirai *et al.*, 1981; Gordon *et al.*, 1985; Tengamnuay and Mitra, 1990), SLS decreased permeation rate of AM when added to PG. Additional studies may be necessary to fully elucidate the effect of SLS for AM.

Table I. Permeation flux and lag time of AM through excised rat skin from 40% PG solutions containing enhancers

Fatty acids	J_s ($\mu g/cm^2/h$)	T_L (h)
None	0.36 ± 0.17	27.1 ± 1.69
LA (5%)	0.93 ± 0.31	27.1 ± 1.01
OA (5%)	3.85 ± 1.47	28.3 ± 0.63
OA (5%)+Lipoid S 100 (2%)	4.62 ± 2.19	29.3 ± 0.53

Data were expressed as the mean ± S.E. (n=3).

Table II. Permeation flux and lag time of AM through excised rat skin from PG solutions containing enhancers

Fatty acids	J_s ($\mu g/cm^2/h$)	T_L (h)
None	0.18 ± 0.09	19.2 ± 4.09
LOA (5%)	23.8 ± 16.3	26.1 ± 1.45
OA (5%)	7.99 ± 2.63	24.5 ± 2.25
OAl (5%)	1.57 ± 0.56	27.6 ± 1.17
LA (5%)	0.52 ± 0.21	24.4 ± 0.37
SLS (1%)	0.02 ± 0.005	17.1 ± 4.30
SLS (1%) + OA (5%)	3.02 ± 0.97	27.2 ± 1.52
SLS (1%) + LOA (5%)	0.41 ± 0.21	24.8 ± 1.51

Data were expressed as the mean ± S.E. (n = 3).

Effect of LOA concentrations on the permeation of AM

AM skin penetration was examined as a function of LOA concentration when PG was used as a vehicle (Fig. 3). The enhancing effect of LOA was increased with concentration up to 5% and a further increase in LOA concentration decreased the AM flux, suggesting a bell-

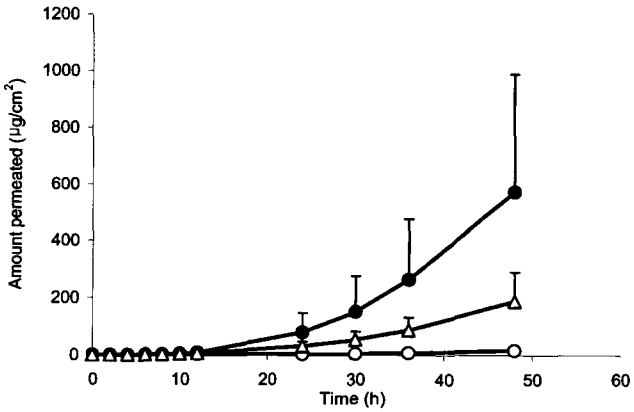

Fig. 3. Effect of the concentration of LOA in PG on the permeation of AM across the excised rat skin. AM was added in a saturating condition in each vehicle composition. Each value represents the mean ± S.E. (n = 3). Key: blank circle, 2 % LOA; filled circle, 5% LOA; blank triangle, 10% LOA.

shaped curve for the enhancing effect. In this study, underlying mechanism for this observation was not directly investigated. Although a further study may be necessary for the elucidation of the mechanism, the reduction of the skin/vehicle partition coefficient of AM appears involved.

Effect of donor doses on the flux of AM

In this study, the effect of drug concentration on the permeation rate of AM was also studied. The composition of the dosing solution was consisted of PG with 5% LOA, with varying concentration of AM (i.e., 0.5, 1 and 2%). As shown in Table III, there was no difference in the permeation rate between 0.5 and 1% which were unsaturated concentrations. On the contrary, at the 2% concentration, a saturated concentration, the flux markedly increased by highly increased thermodynamic activity. Therefore, addition of AM over the saturated concentration may be necessary for the realization of the practical transdermal delivery device for the drug.

Effect of animal species on the flux of AM

To better assess the dependency of the species on the transdermal delivery of AM, the permeation rates were compared for AM in rat and hairless mouse dorsal skins. In hairless mouse skins, the highest flux was achieved when using PG and 5% LOA, similar in composition to that found in rat skin study (Table IV). Interestingly, the flux in mouse skin was 9 times higher than that for rat skins. Lag time markedly decreased in hairless mouse skin compared to rat skin. These results were thought to be mainly due to the difference in skin thickness. Since there exists a significant difference in specie difference in the transdermal transport for AM, a study involving human skins may be necessary to better assess the feasibility of transdermal administration for AM.

Table III. The effect of drug concentration on permeation flux and lag time of AM through excised rat skin from PG solutions containing 5% LOA

Drug concentration (%)	J_s (μg/cm²/h)	T_L (h)
0.5	6.77 ± 3.53	21.8 ± 0.71
1.0	5.80 ± 4.62	22.4 ± 3.01
2.0	23.8 ± 16.4	26.1 ± 1.45

Data were expressed as the mean ± S.E. (n = 3).

Table IV. The effect of animals on the flux and lag time of AM

Species	Fatty acids	J_s (μg/cm²/h)	T_L (h)
Rat	None	0.19 ± 0.09	19.2 ± 4.09
	OA (5%)	7.99 ± 2.63	24.5 ± 2.25
	LOA (5%)	23.8 ± 16.4	26.1 ± 1.45
Hairless mouse	None	1.72 ± 0.51	23.4 ± 0.64
	OA (5%)	188.4 ± 21.2	2.89 ± 0.91
	LOA (5%)	213.3 ± 45.1	6.09 ± 0.09

Data were expressed as the mean ± S.E. (n = 3).

Degradation of AM in skin extracts and homogenates

During skin permeation study of AM, SM or SA as well as AM were detected in the receptor compartment, suggesting a metabolic instability of the drug. Thus, the enzymatic degradation of AM was evaluated using rat skin extract and homogenate. As shown in Fig. 4, the first order degradation rate constants in the epidermal and serosal extracts were 0.011 ± 0.004 h^{-1} and 0.57 ± 0.02 h^{-1}, respectively. As a control, the rate constant was estimated to be 0.028 ± 0.008 h^{-1} in saline, suggesting a chemical instability of the AM in addition to metabolic instability. The faster degradation of AM in serosal extract appeared consistent the protein concentrations found in the epidermal and serosal extracts (i.e., epidermal extract, 14.49 μg/ml; serosal extract, 127.5 μg/ml).

The first order degradation rate and degradation half-life in the rat skin homogenate was calculated to be 6.15 ± 0.14 h^{-1} and 0.11 ± 0.003 h, respectively. Protein concentration of the rat skin homogenate preparation was found to be 1,449 μg/ml. Again, protein concentration in homogenate (i.e., higher by 11.4 fold) and the degradation rate constant (i.e., larger by 10.8 fold) appeared correlated well in the difference in metabolic instability of AM. From these results, AM was thought to be degraded mainly by the enzymes in the dermal and subcutaneous tissues of the skin, regardless of the source of the tissue.

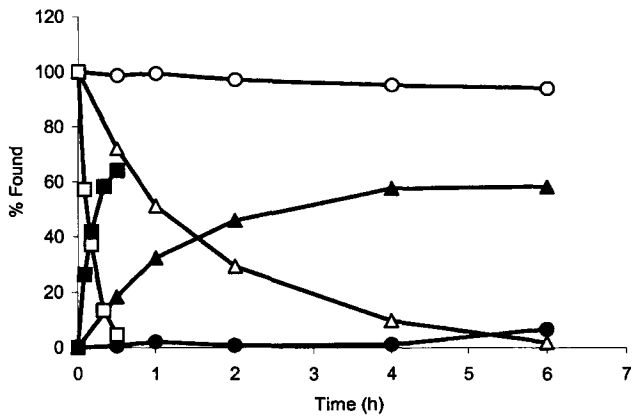

Fig. 4. Degradation of AM and formation of SM in the epidermal and serosal extracts, and homogenates of rat skins. Each value represents the mean of three determinations. Key: blank circle, AM in the epidermal extract; filled circle, SM in the epidermal extract; blank triangle, AM in the serosal extract; filled triangle, SM in the serosal extract; blank square, AM in the skin homogenate; filled square, SM in the skin homogenate.

Taken together, our data indicated that transdermal delivery of AM is feasible when the combination of PG and LOA is used as a vehicle. However, since AM is not metabolically stable, acceptable degradation inhibitors may be necessary to fully realize the transdermal delivery of the drug.

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