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Role of sphingosine synthesis inhibition in transcutaneous delivery of levodopa

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Abstract

The present study was designed to investigate the role of skin sphingosine synthesis inhibition in enhancing the in vitro permeation of levodopa (LD), a hydrophilic drug, across rat skin. β-Chloroalanine (β-CA), a selective inhibitor of serine palmitoyl transferase was used for inhibiting sphingosine synthesis in viable skin. The sphingosine content in viable skin perturbed by acetone treatment and immediately treated with β-CA (600 or 1200 μg/7 cm^2) was significantly less than that of perturbed viable skin after 36 h of treatment ($P < 0.001$). The in vitro permeation of LD across perturbed-β-CA treated skin was significantly greater than that across perturbed skin ($P < 0.001$). This indicates an inverse relationship between in vitro permeation of LD and skin sphingosine content. The systemic delivery of percutaneously applied LD across normal rat skin was negligible. Higher C_{max}, lower T_{max} and maintenance of effective plasma concentration of LD over 28 h was achieved by a single topical application of carbidopa–LD combination (1:4) to perturbed-β-CA treated skin. Hence, skin sphingosine synthesis inhibition can be used as a novel means of enhancing systemic delivery of LD.

Keywords: Skin sphingosine; β-Chloroalanine; Percutaneous permeation; Levodopa

1. Introduction

Levodopa (LD), the first drug of choice in the management of parkinsonism, exhibits a short plasma $t_{1/2}$ of 1.4 h (Robertson et al., 1989). Although, it is absorbed completely after oral administration, 95% of the orally administered dose undergoes decarboxylation in the intestinal lumen and gut wall by dopa decarboxylase to dopamine that does not cross the blood–brain barrier (Rivera-Calimlim et al., 1971). In fact, less than 5% of the oral dose reaches the brain (Granerus et al., 1973). In addition, chronic oral administration of LD may result in altered absorption either due to changes in motility of the GIT or due to competition with dietary amino acids for L-neutral amino acids transport systems (Nutt et al., 1984).

Transdermal route is envisaged to offer an attractive alternative means for systemic delivery of LD. However, LD is a highly hydrophilic drug, exhibiting a log $K_{o/w}$ of -4.7 (Yamaguchi et al.,

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1997). Since the percutaneous permeation of hydrophilic drugs across intact stratum corneum is highly restricted (Okumura et al., 1989), there is a need to increase the percutaneous permeation of LD.

Ceramides, the main polar lipid, constitute a major portion (50%) of intercellular lipid matrix in the stratum corneum (Elias and Menon, 1991). They play an important role in maintaining the excellent barrier properties of skin. Ceramides are synthesized by acetylation of sphingosine with fatty acid. Sphingosine itself is synthesized in the lower layers of viable epidermis following the condensation of palmitoyl CoA with L-serine. This rate limiting step in the synthesis of ceramide is catalyzed by serine palmitoyl transferase (SPT) enzyme. β-Chloroalanine (β-CA) being a selective inhibitor of SPT enzyme (Holleran et al., 1991b), blocks the first step (condensation of palmitoyl CoA with L-serine) in the synthesis of sphingosine, leading to decreased ceramide content in the skin. This is accompanied with the appearance of abnormal lamellar bodies in viable epidermis leading to impaired barrier function. Perturbation by solvent treatment also decreases skin lipid content and makes the skin more permeable to water loss (Behne et al., 2000). However, the influence of perturbation by solvents is a short term effect and the perturbed skin starts regaining its barrier properties within 5 h of perturbation (Holleran et al., 1991a). Hence, alteration of skin ceramide content with β-CA application following solvent perturbation is envisaged to play a vital role in enhancing percutaneous permeation of LD. A similar approach using lipid synthesis inhibitors of ceramide, fatty acid and cholesterol has been demonstrated to enhance the in vitro and in vivo transdermal delivery of lidocaine and caffeine (Tsai et al., 1996).

The present investigation aims at exploring the role of sphingosine synthesis (a precursor for ceramide) inhibition by topical application of β-CA on in vitro and in vivo percutaneous permeation of LD, a polar drug. In addition, the role of co-application of carbidopa (a dopa decarboxylase inhibitor) on the pharmacokinetics of percutaneously delivered LD was investigated.

2. Experimental

2.1. Materials

D-sphingosine and β-chloro-L-alanine were purchased from Sigma Chemicals, USA. LD USP and carbidopa USP were gift samples from Sun Pharma, Baroda, India. All other chemicals were of AR grade.

Albino wistar rats of either sex weighing 175–225 g maintained on a standard laboratory diet and tap water ad libitum were used in the present study.

2.2. Methods

2.2.1. Stability of LD in receptor fluid (phosphate buffer IP, pH 7.4)

The stability of LD was determined in phosphate buffer (PB) IP (pH 7.4) containing formaldehyde (0.1% v/v of 40% v/v solution) or sodium azide (0.05% w/v) as preservatives. The solutions were stored at 37 ± 2 °C and samples (1 ml) were analyzed spectrophotometrically at different intervals till 48 h for λ_{max} and absorbance. The role of sodium sulfite (0.25% and 0.75% w/v) in preventing the oxidation of LD in PB was studied by employing Doty reaction (Doty, 1948).

2.2.2. Selection of solvent and treatment time for sphingosine extraction ability (in vitro)

Epidermis was obtained by soaking the freshly excised whole dorsal skin (hair removed by electric razor) of rats in water (60 °C for 45 s). The underlying dermis was then removed by gentle scraping (Williams and Barry, 1991). The epidermal skin was mounted on a vertical Keshary–Chien diffusion cell and treated with ethanol, 2-propanol or acetone (0.5 ml). The receptor fluid (PB, pH 7.4 maintained at 37 ± 2 °C) was stirred for 5, 10 or 20 min and the skin was removed.

For sphingosine estimation, the epidermal skin was removed from the diffusion cell at the end of the experiment. The portion exposed to the donor/receptor compartment was cut and dried to constant weight. The dried skin pieces were homogenized in potassium PB (pH 7.0) and the homogenate extracted five times successively with

fresh chloroform:methanol (1:2) mixture. Additional chloroform was added followed by addition of 1 M NaCl solution. The extract was then centrifuged and the upper aqueous phase was aspirated and another aliquot of NaCl solution was added and the step repeated. The residual chloroform phase was vacuum dried and then resuspended in 0.1 M KOH in chloroform:methanol (1:2). This mixture was extracted twice with chloroform followed by addition of NaCl solution and then centrifuged. The aqueous layer was aspirated and the chloroform layer obtained was finally vacuum dried. Further derivatization was done with *o*-phthalaldehyde solution (0.5 mg/ml containing 35 boric acid, pH 10.5, 0.05% β-mercaptoethanol). Sphingosine estimation was done by utilizing excitation wavelength of 340 nm and emission wavelength of 455 nm (Sabbadini et al., 1993).

2.2.3. Dose dependent influence of β-CA on sphingosine content in viable skin

The dorsal skin surface of rats was shaved with an electric razor to obtain three patches (7 cm^2). Two patches were perturbed by acetone treatment (0.5 ml, 10 min) alone or acetone treatment followed by immediate application of β-CA (400, 600 or 1200 μg) solution prepared in a mixture (0.25 ml) of propylene glycol:ethanol (7:3). The third patch was not perturbed and served as control. The skin patches were excised by sacrificing the animals (after 5 or 36 h). The skin patches were dried to constant weight and subjected to sphingosine content determination.

2.2.4. Permeation studies (in vitro)

The amount of LD to be loaded in the donor compartment for achieving an effective plasma concentration (C_p) of 1.58 μg/ml (Robertson et al., 1989) was calculated by using the formula, $C_p \times V_d \times K_e$ (1.58 μg/ml × 1.7 l/kg × 0.495/h). Hence, 63.8 mg of LD was used in the donor compartment to obtain a release rate of 1.329 mg/h for 48 h.

2.2.4.1. Selection of donor vehicle for permeation of LD. Acetone-perturbed (0.5 ml, 10 min) and normal epidermal skin were stabilized for 4 h in the diffusion cell and the receptor compartment was refilled with fresh fluid (PB, pH 7.4; sodium azide, 0.05% w/v and sodium sulfite, 0.25% w/v). LD (63.8 mg) dispersed in ethanol, propylene glycol or water (10 ml) was loaded in the donor compartment and sealed with aluminum foil. The entire assembly was positioned in a thermostatically controlled water bath (37 ± 2 °C) and stirred at 300 rpm. Samples (1 ml) were withdrawn at various time intervals through 48 h and immediately analyzed for LD at 280 nm (Gamez et al., 1976). The receptor fluid was replenished with an equal volume of fluid maintained at the same temperature. Flux of LD was calculated from the linear steady-state portion of the plot between cumulative amount of LD permeated vs time.

2.2.4.2. Permeation of LD across perturbed-β-CA treated excised skin. Two patches (7 cm^2), one on either side of spinal cord were prepared by shaving with electric razor. One patch was perturbed by acetone treatment (0.5 ml, 10 min) and served as control. The other patch received acetone treatment followed by immediate application of β-CA (400, 600 or 1200 μg/0.25 ml). The animal was sacrificed after 36 h. The epidermal skin obtained from these excised patches were used for studying the in vitro permeation of LD. Three groups, one for each dose, were used for this study. Each group consisted of six rats.

2.2.5. Pharmacokinetic studies

LD (63.8 mg/ml) dissolved in propylene glycol:ethanol (7:3) mixture was applied to shaved dorsal skin (7 cm^2) of rats. Skin treatment given to five groups, each comprising six animals, can be summarized as: Group I, no treatment (control); Group II, perturbed by acetone treatment (0.5 ml, 10 min); Group III, perturbed + β-CA application (600 μg); Group IV, perturbed + β-CA application (600 μg) + carbidopa (16 mg); Group V, perturbed + β-CA application (600 μg) + carbidopa (6.4 mg). Blood sample (0.25 ml) was obtained from tail vein in heparinized syringe and immediately transferred to eppendroff tubes containing sodium metabisulphite (5 mg). Plasma obtained by centrifugation was analyzed spec-

trofluorometrically with excitation and emission wavelengths of 440 and 470 nm, respectively, for intact LD after coupling it with naphthoresorcinol according to the method reported by Cotler et al. (1976).

3. Results

LD was found to degrade immediately in PB, both with and without formaldehyde (0.1% v/v of 40% v/v solution). Sodium sulfite did not prevent the degradation of LD in presence of formaldehyde. However, when sodium azide (0.05% w/v) was used as preservative, sodium sulfite (0.25% or 0.75% w/v) prevented the degradation of LD in PB (Fig. 1).

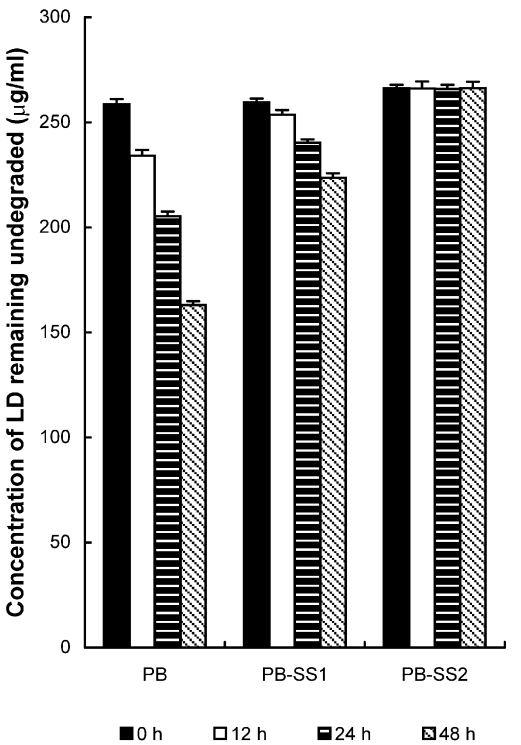

Fig. 1. Concentration of LD (μg/ml) remaining undegraded in different systems. PB: phosphate buffer (pH 7.4); PB-SS1: PB, formaldehyde (0.1% v/v of 40% v/v solution) and sodium sulfite (0.25% w/v); PB-SS2: PB, sodium azide (0.05% w/v) and sodium sulfite (0.25% w/v). Error bars indicate mean ± s.d.

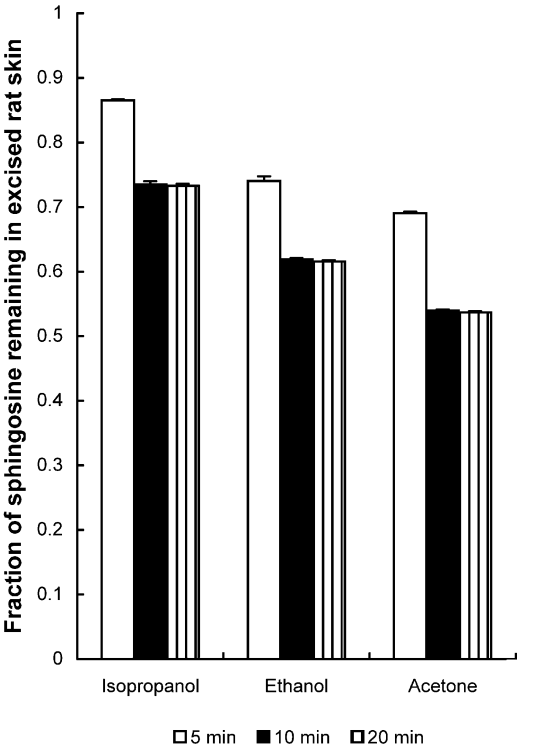

Fig. 2. Fraction of sphingosine remaining in excised rat skin (sphingosine (μg) in solvent perturbed skin/sphingosine (μg) in physiological saline treated skin) after extraction by different solvents following treatment for various time periods. Error bars indicate mean ± s.d.

Fig. 2 shows that there is no significant difference in the amount of sphingosine remaining in skin following treatment with either solvent at 10 or 20 min of treatment time ($P < 0.05$). Among the solvents tested, acetone was found to be most effective in extracting sphingosine from excised skin.

In viable skin, a significant difference was observed in sphingosine content between normal and perturbed skin after 5 h of treatment ($P < 0.02$). However, there was no significant difference in sphingosine content between normal and perturbed viable skin after 36 h of treatment ($P < 0.001$). Application of β-CA at either dose (400, 600 or 1200 μg/7 cm^2) to perturbed viable skin resulted in significant inhibition of sphingosine synthesis till 5 h ($P < 0.001$). But, the decreased sphingosine content of perturbed viable skin was

significantly sustained till 36 h ($P < 0.001$) only by higher doses (600 or 1200 μg/7 cm^2) of β-CA (Fig. 3).

The in vitro permeation enhancement ratio of LD (flux across perturbed skin/flux across normal skin) for various donor vehicles followed the order, ethanol > propylene glycol = water ($P < 0.001$), indicating suitability of ethanol as the donor vehicle for percutaneous delivery of LD (Fig. 4). The enhancement ratio for in vitro permeation of LD using ethanol as donor vehicle across perturbed-β-CA-treated viable skin excised after 36 h shows no significant difference for 600 and 1200 μg/7 cm^2 doses of β-CA ($P < 0.001$). Fig. 5 shows that the enhanced permeation of LD is directly proportional to the sphingosine synthesis inhibition efficacy of β-CA.

Pharmacokinetic studies in rats reveal negligible concentration of LD in plasma following topical application to normal skin. Perturbation followed by β-CA treatment (600 μg/7 cm^2) enhanced the transcutaneous permeation of LD. Co-application of carbidopa with LD (1:4) maintained effective plasma concentration of LD for 28 h (Fig. 6).

4. Discussion

The stability of LD is reported to be influenced by pH and storage temperature (Lund, 1994). Since, the in vitro permeation studies involve the use of PB and a preservative for protecting the excised animal skin from microbial attack, the influence of formaldehyde and sodium azide (preservatives) on stability of LD in PB was investigated. LD degraded immediately in PB alone and in PB containing formaldehyde resulting in formation of black colour. Sodium sulfite, an antioxidant, did not prevent the darkening of LD solution in presence of formaldehyde, probably due to its neutralization by formaldehyde. However, when sodium azide (0.05% w/v) was used as

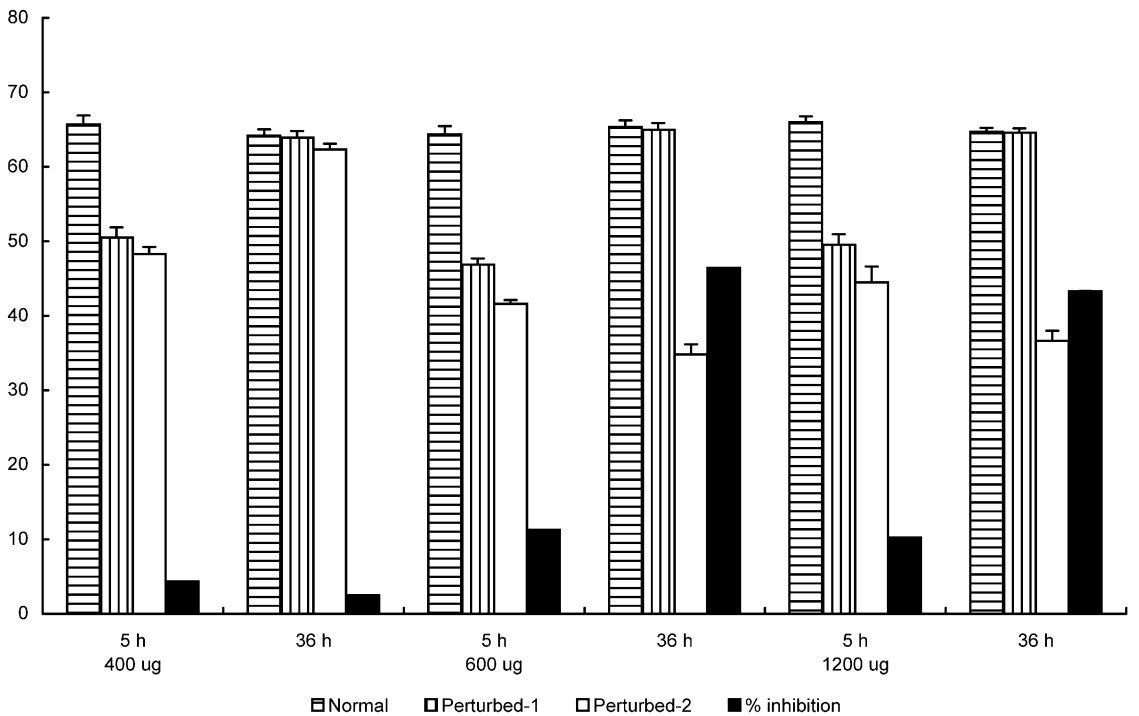

Fig. 3. Dose dependent influence of β-CA on sphingosine content (μg) and its percentage synthesis inhibition after various treatments in viable rat skin excised after 5 or 36 h of treatment. Perturbed-1 represents skin treated with acetone for 10 min; Perturbed-2 represents skin treated with acetone followed by immediate application of β-CA. Error bars indicate mean ± s.d.

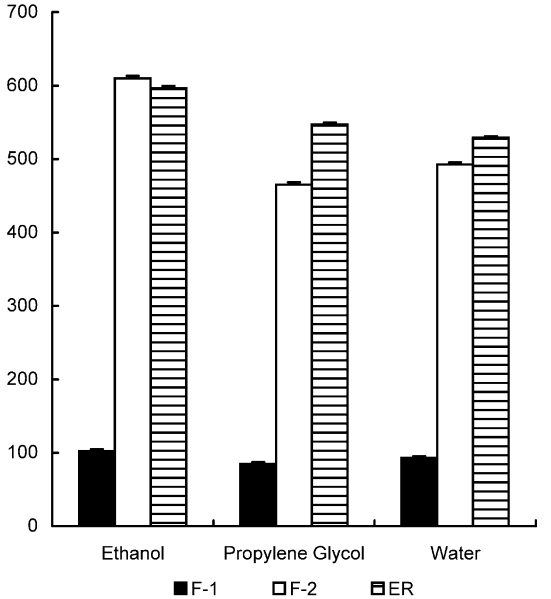

Fig. 4. Flux of LD (μg/cm^2/h) across normal rat skin (F-1), acetone perturbed rat skin (F-2) and in vitro permeation enhancement ratio (ER) of LD using different donor vehicles. Values of ER shown in the figure represent actual value of ER × 100. Error bars indicate mean ± s.d.

preservative (Catz and Friend, 1990), sodium sulfite (0.25% or 0.75% w/v) prevented the oxidation of LD in PB till 48 h at 37 ± 2 °C (Fig. 1).

There was insignificant difference in the amount of sphingosine remaining in skin after 10 or 20 min of treatment with either solvent ($P < 0.05$). Acetone possessing lower dielectric constant (21.5) than ethanol (25) extracted greater amount of sphingosine from excised skin because of its ability to form stronger hydrogen bonds with sphingosine molecule due to greater negative charge on its ketonic oxygen. 2-Propanol with the lowest dielectric constant (13.8) produced least sphingosine extraction (Fig. 2). This indicates that a treatment of 10 min with acetone (0.5 ml) is optimum for extracting sphingosine from excised rat skin.

Fig. 3 shows that the sphingosine content in acetone-perturbed viable skin is significantly different than the normal skin after 5 h of perturbation ($P < 0.02$). However, after 36 h of perturbation, the sphingosine content in perturbed skin returns to insignificantly different level as compared to normal skin ($P < 0.001$). This is because of abrupt increase in sphingosine synthesis after 3 h of perturbation effect in a bid to restore the barrier status of skin, which ultimately normalizes by 24 h (Holleran et al., 1991a).

Hence, for enhancing transcutaneous permeation of LD, it was felt necessary to sustain the low sphingosine content of perturbed skin for a longer time period. β-CA is reported to irreversibly inhibit serine palmitoyltransferase, the enzyme that catalyzes the condensation of palmitoyl CoA with serine molecule, thus leading to inhibition of the first step in ceramide biosynthesis (Medlock et al., 1988). However, β-CA exhibits limited permeability across intact skin (Holleran et al., 1991b). The percentage of sphingosine synthesis inhibition [1 − (sphingosine content (μg) in acetone perturbed-β-CA treated skin/sphingosine content (μg) in acetone perturbed skin) × 100] at the end of 36 h (Fig. 3) followed the order,

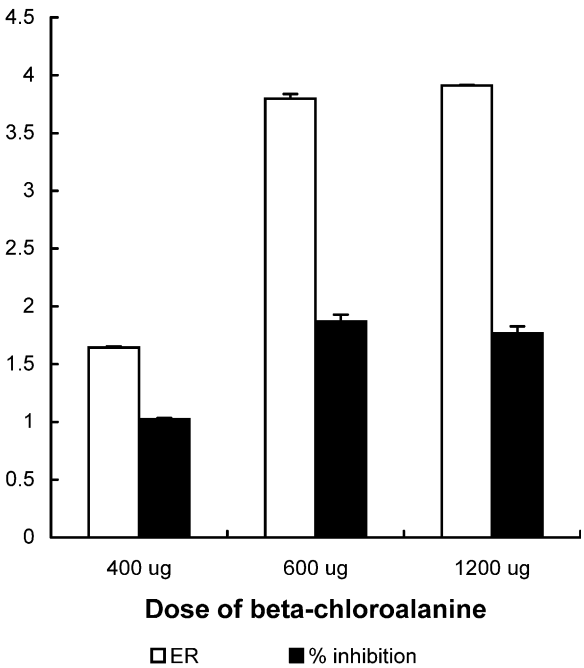

Fig. 5. In vitro permeation enhancement ratio (ER) of LD and percentage sphingosine synthesis inhibition in acetone perturbed-β-CA treated viable rat skin excised after 36 h of treatment. Error bars indicate mean ± s.d.

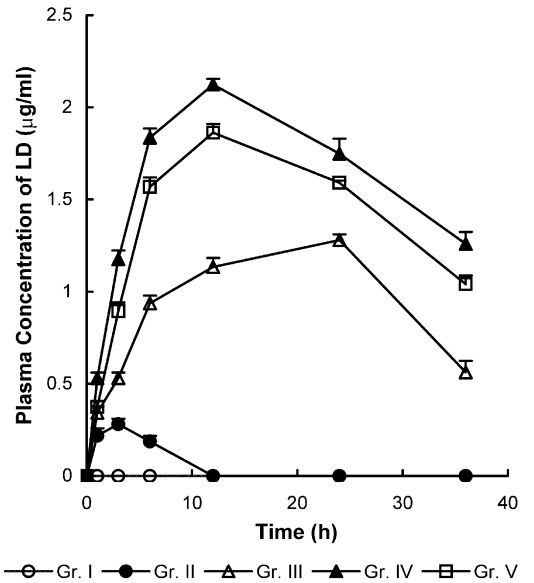

Fig. 6. Systemic delivery of percutaneously applied LD in rats after various treatments. The treatment given to different groups is: Group I: normal skin + LD; Group II: acetone perturbed skin + LD; Group III: acetone perturbed skin + β-CA (600 μg) + LD; Group IV: acetone perturbed skin + β-CA (600 μg) + LD + carbidopa (16 mg); Group V: acetone perturbed skin + β-CA (600 μg) + LD + carbidopa (6.4 mg). Error bars indicate mean ± s.d. of six determinations.

$1200 = 600 > 400$ μg/7 cm^2 ($P < 0.001$). This indicates that a dose of 600 μg/7 cm^2 is capable of inhibiting sphingosine synthesis in perturbed viable skin till 36 h after treatment.

In addition, the permeation of LD across perturbed-β-CA treated viable skin excised after 36 h was insignificantly different for β-CA dose of 600 and 1200 μg/7 cm^2 ($P < 0.001$). It is noteworthy, that the enhancement ratio for in vitro permeation of LD using ethanol as vehicle is directly proportional to the percentage of sphingosine synthesis inhibition by β-CA in viable skin (Fig. 5). However, the actual value of in vitro enhancement ratio of LD using ethanol vehicle across skin excised immediately after perturbation (Fig. 4) is significantly greater ($P < 0.001$) than that across perturbed-β-CA treated skin excised after 36 h (Fig. 5). This is due to the fact that although, β-CA inhibits synthesis of sphingosine, the synthesis of other skin constituents like fatty acid (Ottey et al., 1995) and cholesterol (Feingold et al., 1990) as a response to perturbation of viable skin is accelerated. Therefore, the role of these skin constituents in restoring the barrier status of skin, thereby restricting the permeation of LD cannot be totally ruled out.

Fig. 6 shows negligible plasma concentration of LD when it was applied to normal skin (Group I). Although, perturbation of skin increased the systemic delivery of LD, no drug was detectable in plasma after 12 h (Group II), possibly, because sphingosine level starts returning to basal value after 3 h of perturbation. The systemic delivery of LD was further enhanced across perturbed-β-CA-treated skin (Group III). However, LD concentration still did not reach the effective plasma concentration of 1.58 μg/ml (Robertson et al., 1989). This is perhaps due to the decarboxylation of LD in blood (Lovenberg et al., 1962; Sudo et al., 1998).

The plasma concentration of LD was significantly higher ($P < 0.001$) across perturbed-β-CA-treated skin when LD was applied in combination with carbidopa in a ratio of 4:1 (Group IV) as compared to that obtained by using a combination of 10:1 (Group V). Although, effective LD plasma concentration were achieved in both cases, the effective level was sustained for longer duration (28 h) by the 4:1 combination of LD and carbidopa. However, a time lag of 3 h was observed for achieving effective plasma concentration.

Sudo et al. (1998) were able to obtain peak plasma concentration of LD in rats within 1 h of cutaneous attachment of LD hydrogel containing 40% ethanol and 2% L-menthol. However, it is interesting to note that the peak plasma concentration of LD achieved was 40 ng/ml which is significantly lower than the required effective therapeutic level of 1.58 μg/ml (Robertson et al., 1989). In addition, the presence of 40% ethanol in the formulation (Sudo et al., 1998) seems to be too high with regards to adverse effect on skin (Ohara et al., 1994).

The present investigation reveals that the required therapeutic plasma level of LD can be sustained over 28 h after a single topical application of LD by employing β-CA. However, further studies aimed at investigating the role of other

skin constituents/formulation additives for decreasing the time lag for achieving effective plasma level are advocated. Nevertheless, results of the present investigation provide a novel approach for enhancing the systemic delivery of LD.

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