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Transcutaneous Delivery of Levodopa: Enhancement by Fatty Acid Synthesis Inhibition

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Abstract: The present investigation aimed at evaluating the role of fatty acid synthesis inhibition in enhancing transcutaneous delivery of levodopa (LD). Rat epidermis was treated with ethanol and various doses of cerulenin (an inhibitor of fatty acid synthase enzyme system) for reducing the normal level of fatty acids. Calcium chloride (0.1 mM) and/or verapamil (1 μM) were coapplied to cerulenin treated skin in order to modulate duration of epidermal perturbation. These treated skin portions were used for estimation of altered triglyceride content (an indicator of fatty acid synthesis), differential scanning calorimetry (DSC) analysis, and in vitro permeation of LD. Plasma concentration of LD was monitored in rats following topical application of various transdermal formulations. Application of cerulenin (0.1 or 0.15 mM/7 cm^2) to viable rat skin inhibited approximately 60% triglyceride synthesis with respect to control at 2 h. Coapplication of calcium chloride (0.1 mM) significantly increased this inhibition, whereas verapamil application reduced this effect. The decrease in triglyceride content reduced the enthalpy of the lipid endothermic transition. The in vitro permeation of LD was enhanced 3-fold across skin excised after treatment with cerulenin. LD did not permeate across normal skin. The effective plasma concentration (C_{eff}) of LD was achieved within 3 h and maintained till 10 h by a single topical application of a carbidopa−levodopa combination (1:4) to ethanol-perturbed cerulenin-treated skin. Coapplication of calcium chloride reduced the time lag to achieve C_{eff} to 2 h and maintained it till 24 h. A single transdermal LD (64 mg) patch formulated with calcium chloride (0.1 mM) and cerulenin (0.1 mM) dissolved in a propylene glycol:ethanol (7:3) mixture seems to offer a noninvasive approach for transcutaneous delivery of levodopa.

Keywords: Levodopa; transcutaneous delivery; skin fatty acid; cerulenin; differential scanning calorimetry

Introduction

Levodopa (LD) is widely used for the management of parkinsonism. Its short plasma half-life (1.4 h) necessitates oral administration thrice a day. Orally administered LD causes variable and unreliable clinical responses because of its erratic oral absorption and first-pass metabolism. The oral bioavailability of LD alone is estimated to be 5%, and less than 1% of the orally administered dose reaches the brain.[1] Further, the patients of Parkinson's disease are occasionally geriatric and tend to have dementia and dysphagia. These types of patients cannot be expected to comply with oral administration. Strict maintenance of constant plasma LD concentration is required to prevent akinesia and dyskinesia in Parkinson's patients.[2] Although this objective can be achieved by intravenous administration, the iv route has very low patient compliance.

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Therefore, transdermal application is envisaged to offer a noninvasive, patient-compliant route for sustained systemic delivery of LD. However, LD is a polar drug with a log $K_{o/w}$ of −4.7 and exhibits less percutaneous permeation.[3] Since the intact skin provides an excellent permeability barrier for percutaneous delivery of polar drugs, there is a need to increase the percutaneous permeation of LD.

The permeability barrier properties of the skin are mediated by a series of lipid multilayers, enriched in ceramides, cholesterol, and fatty acids segregated within the stratum corneum interstices.[4] Fatty acids (as triglycerides) constitute a major portion (∼50%) of the epidermal nonpolar lipids.[5] There are two key enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), that are known to be involved in the biosynthesis of fatty acid.[6] ACC carboxylates acetyl-CoA to produce malonyl-CoA, which is converted to free palmitate by FAS.[7] The synthesis of fatty acids in skin has been reported to be inhibited by 5-(tetradecyloxy)-2-furan-carboxylic acid (TOFA) or cerulenin (CN). TOFA has been found to get esterified to CoA to produce TOFA-CoA, which profoundly inhibits ACC,[8] whereas cerulenin noncompetitively inhibits the FAS system.[9]

Topical application of organic solvents or tape stripping removes lipids from the stratum corneum, thereby disrupting the permeability barrier. These perturbations initiate a sequence of events, which include an increased epidermal synthesis of cholesterol, sphingolipid, and fatty acid.[10−12] This rapidly restores lipids to the stratum corneum resulting in normalization of barrier function. Few reports indicate the role of lipid synthesis inhibitors[13−15] in enhancing the percutaneous delivery of drugs. In addition, calcium and potassium ions are important regulators of barrier homeostasis.[16] Therefore, it can be hypothesized that combining calcium ions with lipid synthesis inhibitors shall further enhance drug permeation.

The present study is designed to investigate the influence of topical application of cerulenin, a selective inhibitor of the fatty acid synthase system (enzyme system that controls the synthesis of fatty acids), to ethanol-perturbed viable skin in increasing the percutaneous permeation of LD. The possible role of calcium chloride in delaying the normalization of triglycerides in perturbed epidermis and enhancing the trancutaneous delivery of LD was also studied. Furthermore, the thermotropic behavior of cerulenin-treated rat epidermis was studied.

Experimental Section

Materials. Cerulenin was purchased from Sigma Chemicals. Levodopa was purchased from SRL, India. Carbidopa was a generous gift from Sun Pharma, Baroda, India. Verapamil was a gift sample from Torrent Pharmaceuticals, Ahmedabad, India. Triglyceride estimation kits (Enzokit) were purchased from Ranbaxy Laboratories Ltd., New Delhi, India. All other chemicals were of AR grade. Albino Wistar rats of either sex weighing 190−210 g were used in this study. The animals were housed under standard laboratory conditions and had free access to food and water. The experimental protocols were approved by the Departmental Ethical (Animal) Committee.

Methods

Dose Dependent Influence of Cerulenin on Triglyceride Content in Rat Epidermis. Three patches (7 cm^2) were prepared on the dorsal skin surface of rats by shaving with an electric razor. The treatments to these patches were given 24 h after shaving. One patch was left unperturbed (control). The second patch was perturbed for 10 min with ethanol

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(0.5 mL). The third patch was perturbed by ethanol followed by immediate application of CN (0.05, 0.1, 0.15 mM/0.25 mL) solution prepared in a propylene glycol:ethanol mixture (7:3). Treated skin patches were excised by sacrificing the animals after 2, 4, 6, 12, 24, 36, or 48 h, and epidermal sheets were obtained by immersing full thickness skin patches in water at 60 °C for 45 s.[17] These epidermal sheets were dried to constant weight and subjected to total lipid extraction according to the procedure described earlier.[18] Triglyceride content in these extracts was determined by using triglyceride estimation kits.

Differential Scanning Calorimetric (DSC) Analysis. DSC analysis (ambient to 125 °C, 1 °C/ min) was carried out on both untreated epidermal sheets and those obtained from excised viable skin treated with 0.1 mM CN (Mettler Toledo Star System, 821 E, Switzerland). The epidermis was separated from the whole skin excised at different time intervals of treatment. Samples of dried epidermal sheets were hydrated over a saturated sodium chloride solution (75% relative humidity at 25 °C) for 3 days prior to DSC analysis. All DSC curves were evaluated especially with regard to the phase transition enthalpies (peak areas) and peak maximum temperatures (T_m) of lipid-phase transitions near 70 °C. The percentage reduction in enthalpy (ΔH, J/g) was calculated by using the formula 100 − {[(ΔH of endotherm due to CN treatment)/(ΔH of endotherm in untreated epidermis)]×100}. All experiments were carried out in triplicate.

In Vitro Permeation Studies of LD across Ethanol-Perturbed CN-Treated Excised Rat Epidermal Skin. Two patches (7 cm^2) were prepared one on either side of the spinal cord, by shaving with an electric razor. The treatments to these patches were given 24 h after shaving. One patch was left unperturbed and served as control. The other patch received ethanol treatment followed by immediate application of CN (0.05, 0.1, 0.15 mM/0.25 mL) solutions prepared in a propylene glycol:ethanol mixture (7:3). The animals were sacrificed after 2, 4, 6, 12, 24, 36, or 48 h of CN application. Epidermal skins obtained from these excised patches were used for studying the in vitro permeation of LD using vertical Franz glass diffusion cells. Epidermal sheets were first stabilized for 4 h in the diffusion cell. The receptor compartment was refilled with fresh phosphate buffer IP (pH 7.4) containing sodium azide (0.05% w/v) as preservative, sodium sulfite (0.25% w/v) as an antioxidant, and PEG 400 (10% v/v) as solubilizing agent and stirred at 300 rpm. The donor compartment consisted of LD (64 mg) dispersed in a propylene glycol:ethanol (7:3) mixture (2 mL) containing 0.25% w/v of sodium sulfite as an antioxidant. The entire cell assembly was securely positioned in a thermostatically controlled water bath maintained at 37 ± 2 °C. Samples (0.5 mL) were withdrawn at various time intervals through 48 h and immediately analyzed spectrophotometrically for the amount of LD permeated at 280 nm.[18] An equal volume of fresh phosphate buffer containing sodium sulfite and sodium azide maintained at 37 ± 2 °C was replaced into the receptor compartment after each sampling. Flux (μg/cm^2/h) was calculated from the slope of the steady-state portion of the graph of cumulative amount of LD permeated vs sampling time.

Influence of Calcium Ions and Verapamil on Fatty Acid Recovery in Viable Rat Skin and in Vitro LD Flux across Excised Rat Skin. Seven groups, one corresponding to each time point, were used. Each group comprised three animals. Two patches were prepared on the dorsal side of each rat. One patch received CN (0.1 mM) treatment with calcium chloride (0.1 mM) to ethanol-perturbed skin. Another patch received verapamil (1 μM) treatment along with CN (0.1 mM) and calcium chloride (0.1 mM) to ethanol-perturbed skin. These solutions were prepared in 0.25 mL of a propylene glycol:ethanol (7:3) mixture. The animals were sacrificed at 2, 4, 6, 12, 24, 36, or 48 h of treatment. The treated skin patches were excised and used for studying the in vitro permeation of LD using vertical Franz glass diffusion cells or dried to constant weight and analyzed for triglyceride content according to the procedure described above.

Pharmacokinetic Studies. An adhesive transdermal patch (7 cm^2) was prepared by using adhesive surgical tape, a plastic ring, and a polyethylene backing membrane. LD (64 mg) dispersed in 0.25 mL of a propylene glycol:ethanol (7:3) mixture was loaded into this cavity. Treatment given to various groups, each consisting of three animals, can be summarized as follows: group I, normal skin + LD; group II, ethanol perturbation (0.5 mL) + LD; group III, ethanol perturbation + CN (0.1 mM) + LD; group IV, ethanol perturbation + CN (0.1 mM) + LD + carbidopa (16 mg); group V, ethanol perturbation + CN (0.1 mM) + LD + carbidopa (16 mg) + calcium chloride (0.1 mM). Blood samples (0.25 mL) were withdrawn from the tail vein in heparinized syringes at specified time intervals and centrifuged at 2500 rpm for 10 min at 4 °C. An equal volume of trichloroacetic acid (4% w/v) was added to the separated plasma and again centrifuged at 2500 rpm for 10 min. This deproteinized plasma was collected and stored at −20 °C until analyzed for intact LD spectrofluorometrically with excitation at 440 nm and emission at 470 nm after being coupled with naphthoresorcinol.[19]

Statistical Analysis. Values were expressed as mean ± SD. The data was analyzed using analysis of variance (ANOVA) followed by studentized range test.

Results

Topical application of cerulenin (0.1 mM) to ethanol-perturbed viable rat skin produced 60% inhibition of tri-

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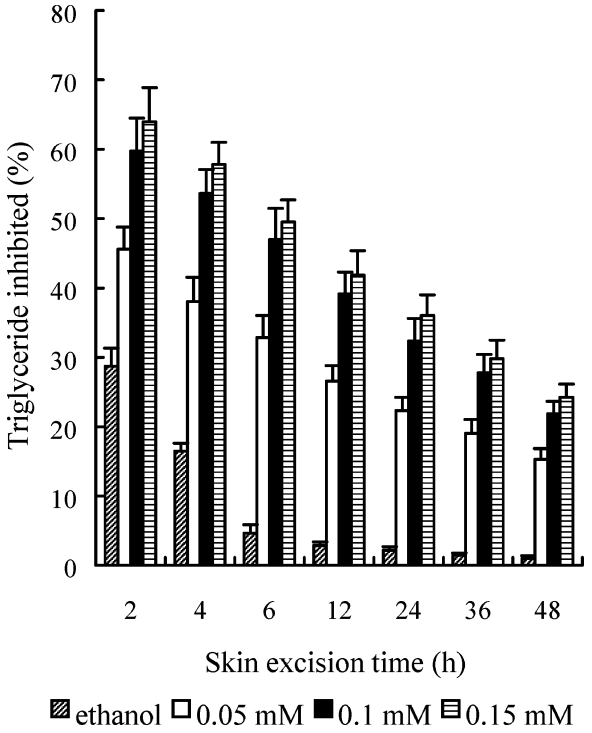

Figure 1. Influence of ethanol or different doses of cerulenin on triglyceride content in viable rat skin. Error bars indicate mean ± SD of three experiments.

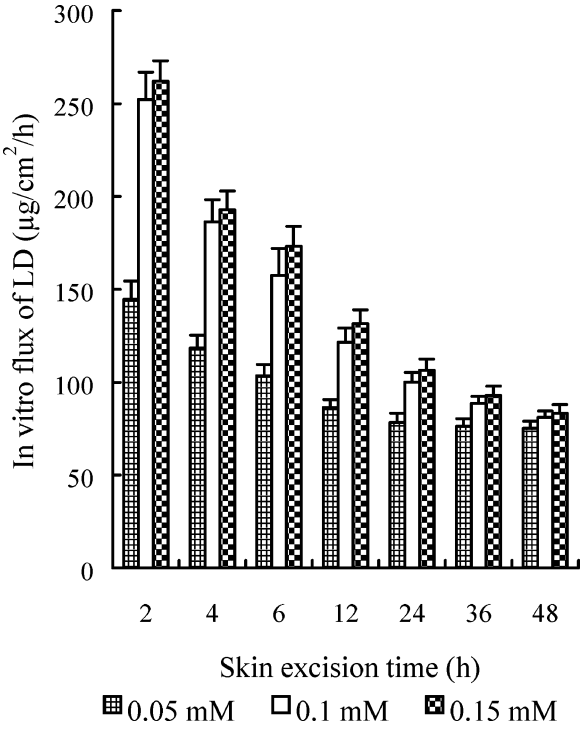

Figure 2. In vitro permeation of LD across ethanol-perturbed viable rat skin treated with different doses of cerulenin. Error bars indicate mean ± SD of three experiments.

glyceride synthesis within 2 h of treatment. At 48 h, 20% synthesis of triglyceride was still inhibited. There was no significant improvement ($p < 0.05$) in inhibition of triglyceride synthesis {1 − [(triglyceride content remaining in skin after CN treatment)/(triglyceride content in normal skin)]×100} after application of a higher dose (0.15 mM) of cerulenin (Figure 1).

Figure 2 shows that the in vitro permeation of LD across ethanol-perturbed CN-treated viable skin excised after different time periods was insignificantly different for 0.1 mM and 0.15 mM/7 cm^2 dose of CN ($p < 0.05$). A direct correlation was observed between percent triglyceride inhibited at various time periods by 0.1 mM of cerulenin and in vitro flux of LD across these excised portions (Figure 3). CN treatment of ethanol-perturbed epidermis decreased the enthalpy of endothermic transition associated with lipids (Figure 4), and this reduction in ΔH was positively correlated with the triglyceride synthesis inhibited (Figure 5). Coapplication of calcium chloride (0.1 mM) and CN to viable skin inhibited significantly more ($p < 0.05$) triglyceride as compared to only CN treatment. The in vitro flux of LD across calcium chloride treated skin was significantly ($p < 0.05$) more than that across ethanol-perturbed CN-treated epidermis (Figure 6).

Pharmacokinetic studies in rats revealed negligible concentration of LD in plasma following topical application to normal skin. However, when LD was applied in combination with carbidopa (4:1) to skin treated with CN, the effective plasma concentration (C_{eff}) of LD was achieved within 3 h and maintained till 10 h. The duration of activity was

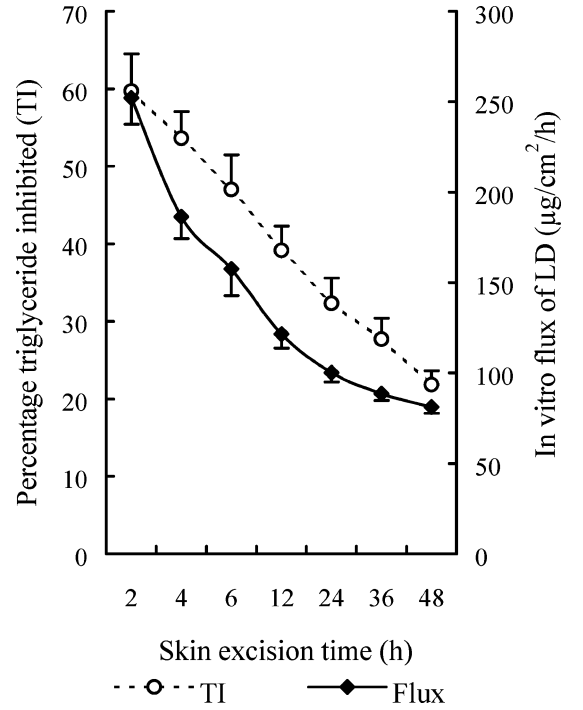

Figure 3. Correlation of triglyceride inhibited (%) and in vitro flux of LD in ethanol-perturbed CN-treated (0.1 mM) rat skin. Error bars indicate mean ± SD of three experiments.

increased to 24 h when calcium chloride (0.1 mM) and CN (0.1 mM) were used in combination. In addition, a significantly higher C_{max} and lower T_{max} ($p < 0.05$) were achieved (Figure 7).

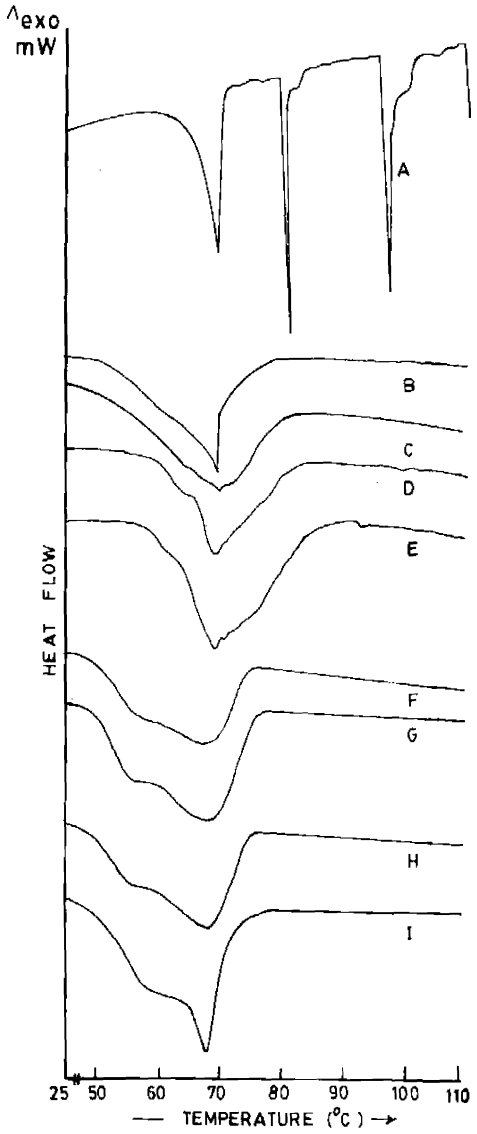

Figure 4. DSC thermograms of excised rat epidermis obtained from viable skin after (A) no treatment; (B) treatment with ethanol; (C) treatment with propylene glycol:ethanol (7:3) for 48 h; or (D−I) treatment with ethanol + cerulenin (0.1 mM) for (D) 2 h, (E) 4 h, (F) 6 h, (G) 12 h, (H) 24 h, or (I) 48 h. All dried epidermal sheets were hydrated at 75% relative humidity prior to thermal analysis. Experiments were carried out in triplicate.

Discussion

It is known that the removal of epidermal lipids by ethanol treatment results in acute disruption of the epidermal permeability barrier status. Although ethanol treatment was found to inhibit 30% of triglyceride synthesis in viable skin at 2 h, its perturbation effect weaned off after 5 h of treatment (Figure 1). These results are in consonance with the findings of Grubauer et al.[11] where acetone treatment increased epidermal fatty acid biosynthesis approximately 3-fold over control at 1−4 h, followed by a rapid decline 5 h after disruption, and returned to normal after 12 h resulting in epidermal barrier repair. Therefore, CN, an inhibitor of the fatty acid synthase enzyme system, was used for delaying fatty acid synthesis in viable rat skin.

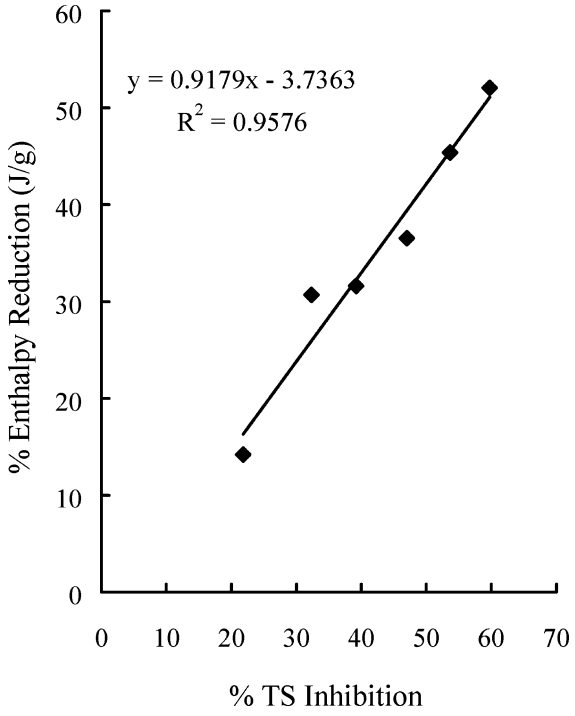

Figure 5. Correlation between endothermic enthalpy (ΔH) reduction and triglyceride synthesis (TS) inhibition. The values represent averages of three experiments.

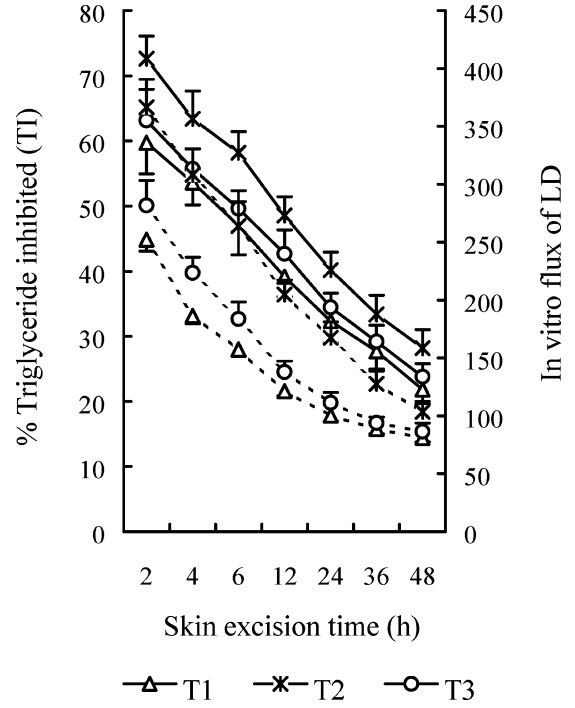

Figure 6. Influence on percent triglyceride inhibited (solid lines) and in vitro permeation of LD across rat epidermis (broken lines) excised after various treatments [T1, ethanol + CN (0.1 mM); T2, ethanol + CN + calcium chloride (0.1 mM); T3, ethanol + CN + calcium chloride + verapamil (1 μM)]. Error bars indicate mean ± SD of three experiments.

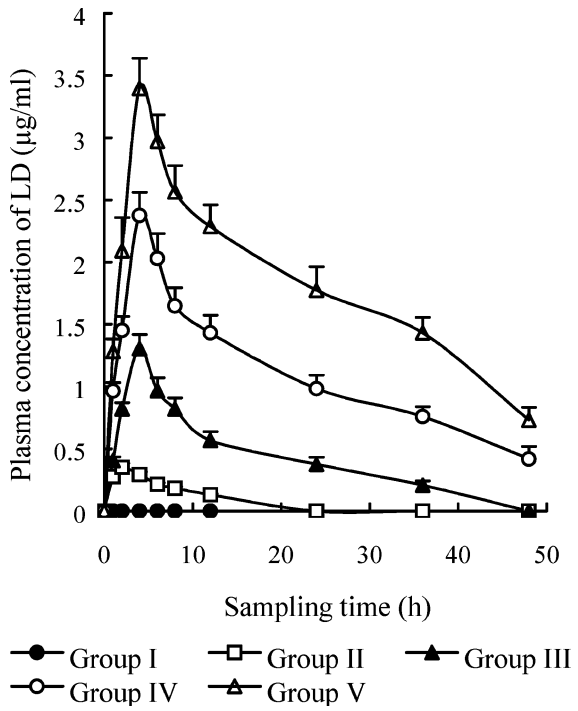

Figure 7. Systemic delivery of percutaneously applied LD in rats after various treatments. The treatment given to different groups was as follows: group I, normal skin +LD (64 mg); group II, ethanol perturbation (0.5 mL) + LD; group III, ethanol perturbation + CN (0.1 mM) + LD; group IV, ethanol perturbation + CN + LD + carbidopa (16 mg); group V, ethanol perturbation + CN + LD + carbidopa (16 mg) + calcium chloride (0.1 mM). Error bars indicate mean ± SD of three experiments.

CN was dissolved in a 7:3 mixture of propylene glycol: ethanol. Propylene glycol has been reported not to produce any significant effect on enthalpy and lipid transition temperature.[17,20] Even ethanol at low concentration (30% w/w) has been reported to increase lipid fluidity only at the polar interface of a lipid bilayer[21] without causing any overall alteration in the degree of stratum corneum lipid−alkyl chain interaction.[22] Further, FTIR studies have ruled out lipid extraction by 66% v/v ethanol.[23] In addition, treatment of porcine skin with either propylene glycol or its combination with ethanol (80:20) has been found to produce an insignificant effect on both peak height and peak area of asymmetric and symmetric C−H stretching absorbance as evidenced by FTIR studies.[24] Hence, the reduced fatty acid synthesis leading to a lower content of triglyceride in CN-treated portions of epidermis (Figure 1) can be attributed to the influence of only CN and not to the vehicle. These findings indicate an insignificant influence of a propylene glycol:ethanol (7:3) mixture on epidermal lipid extraction.

A single topical application of CN (0.1 mM) was able to inhibit epidermal triglyceride by ∼60% in ethanol-perturbed rat skin. The percentage triglyceride inhibited {1 − [(triglyceride content remaining in skin after CN treatment)/(triglyceride content in normal skin)]×100]} at the end of 48 h followed the order 0.15 = 0.1 > 0.05 mM/7 cm^2 ($p < 0.05$). This indicated that a dose of 0.1 mM/7 cm^2 was capable of inhibiting fatty acid synthesis in ethanol-perturbed viable skin till 48 h after treatment (Figure 1).

Maximum in vitro permeation of LD was found across skin excised after 2 h of CN treatment (Figure 2). This seems to be because fatty acid synthesis in the epidermis begins shortly (within 1−2 h) after barrier disruption[11,25] and this was effectively inhibited by CN. The enhancement ratio indicated 3-fold greater in vitro permeation of LD across ethanol-perturbed epidermis treated with CN compared to normal skin. A direct correlation was observed between percent triglyceride inhibited at various time periods by 0.1 mM of CN and in vitro permeation of LD across these excised portions (Figure 3).

The thermogram of untreated rat epidermis exhibited three sharp endothermic transitions at 69.25, 80.45, and 96.53 °C (Figure 4A). Bentley et al.[26] have ascribed these endotherms to lipids, lipid−protein complex, and protein transition at 73, 87, and 104 °C, respectively. Minor differences in the peak transition temperatures observed in our study might have arisen due to lower hydration level (75% relative humidity) compared to that (97% relative humidity) employed by Bentley et al. Only one endothermic transition near 70 °C was observed in the thermogram of viable rat epidermis excised after 48 h of treatment with ethanol or a propylene glycol:ethanol (7:3) mixture (Figure 4B,C).

Thermograms of epidermis obtained from excised portions of viable skin treated with a combination of ethanol and CN (0.1 mM) also exhibited only one endothermic transition near 70 °C (Figure 4D−I). The absence of lipid−protein and protein transitions from ethanol-treated and propylene glycol: ethanol (7:3) treated rat epidermis can be ascribed to their

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dehydrating effects on stratum corneum.[17,20] Maximum reduction in ΔH (%) of lipid endotherm was found at 2 h that decreased with time elapsed due to slow normalization of fatty acid synthesis after treatment with CN. However, this reduction in ΔH (%) of lipid endotherm was directly correlated with the percent triglyceride synthesis inhibited (Figure 5). It is important to note that, although the epidermal contents of the two important lipids ceramide and cholesterol are also known to be reduced by ethanol,nevertheless in the absence of their synthesis inhibitors, their levels normalize within 12−24 h.[12] Hence, the observed alteration of lipid endothermic enthalpy seems to be predominantly due to CN-induced reduction in fatty acid synthesis. These results coupled with the observation of direct correlation between in vitro permeation and percent triglyceride inhibition (Figure 3) strongly suggest significant contribution of fatty acids in the epidermal lipid milieu in impeding the permeation of LD.

The application of calcium chloride (0.1 mM) to ethanol-perturbed CN-treated viable skin produced significantly more inhibition of fatty acid synthesis ($p < 0.05$). As a result these excised portions were more permeable to LD as compared to ethanol-perturbed CN-treated viable skin. A direct correlation between fatty acid synthesis inhibited and in vitro permeation of LD across similarly treated epidermis is evident in Figure 6. However, coapplication of verapamil (1 μM) reduced the calcium-induced inhibition of barrier recovery, and these epidermal portions were less permeable to LD. Hence, it appears that the presence of excessive extracellular Ca^{2+} delays normalization of perturbed lipid levels and verapamil reduces this inhibition by blocking the entry of Ca^{2+} ions into the cytosol of epidermal cells. This appears to be because the efflux of Ca^{2+} from epidermis probably gives a signal for normalization of lipid contents and barrier status of perturbed skin.[16]

The pharmacokinetic studies on systemic delivery of percutaneously applied LD were conducted in Wistar rats following various treatments (Figure 7). LD did not permeate across normal skin (group I), indicating excellent barrier function of intact skin for water-soluble drugs. A very small amount was found to permeate into the systemic circulation when only LD was applied to ethanol-perturbed skin. In addition, no drug was detectable after 12 h of ethanol perturbation (group II). This is probably because the lipid synthesis is completely restored to basal value within 24 h of barrier perturbation.[12] Further, the possibility of metabolism by dopadecarboxylase enzyme present in blood cannot be ruled out. Although CN (0.1 mM) treatment to ethanol-perturbed skin enhanced the systemic delivery of LD (group III), the effective plasma concentration (C_{eff}) of 1.58 μg/mL was not achieved. This is perhaps due to the degradation of LD in vivo by dopadecarboxylase enzyme. Therefore, carbidopa was used to inhibit dopadecarboxylase for increasing the systemic level of intact LD. It is noteworthy that a significantly higher ($p < 0.05$) plasma concentration of LD was achieved across ethanol-perturbed CN-treated skin when carbidopa (dopadecarboxylase inhibitor) was coapplied with LD in a ratio of 1:4 (group IV). The C_{eff} was achieved within 3 h and maintained till 10 h with this treatment. The duration of activity (>1.58 μg/mL) was increased to 24 h when calcium chloride (0.1 mM) and CN (0.1 mM) were used in combination (group V). In addition, a significantly higher C_{max} (3.4 μg/mL) and lower T_{lag} (<2 h) were achieved ($p < 0.05$).

Sudo et al.[27] were able to obtain LD peak plasma concentration of only 40 ng/mL following cutaneous attachment of hydrogel containing ethanol (40%) and menthol (2%). The results of the present investigation reveal that the systemic delivery of LD can be significantly enhanced and maintained above the C_{eff} for 24 h following a single application of a transdermal patch containing fatty acid synthesis inhibitor. Further, studies using a combination of ceramide, cholesterol, and fatty acid synthesis inhibitors will perhaps further help in reducing time lag and increasing duration of action of LD. Nevertheless, the results indicate a feasible approach for noninvasive systemic delivery of levodopa.

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