Lipid Synthesis Inhibitors: Effect on Epidermal Lipid Conformational Changes and Percutaneous Permeation of Levodopa

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ABSTRACT

A combination of lipid synthesis inhibitors was used to enhance the in vitro and in vivo permeation of levodopa (LD) across rat epidermis, and their influence on epidermal lipids was investigated using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Rat epidermis was treated with ethanol and a combination of atorvastatin (750 μg/7 cm^2), cerulenin (20 μg/7 cm^2), and β-chloroalanine (600 μg/7 cm^2) for sustaining the reduced content of epidermal cholesterol, fatty acids (as triglycerides), and ceramide (as sphingosine), respectively, in viable rat skin. This treatment resulted in significant ($P < .05$) synthesis inhibition of skin lipids up to 48 hours and 6-fold enhancement in the in vitro permeation of LD. The effective plasma concentration of LD was achieved within 1 hour and maintained over 48 hours after topical application to rat epidermis treated with a combination of these lipid synthesis inhibitors. ATR-FTIR studies of inhibitor(s)-treated rat epidermis revealed a significant decrease ($P < .05$) in peak height and area for both asymmetric and symmetric C-H stretching absorbances, suggesting extraction of lipids. However, an insignificant ($P < .05$) shift in the frequency of these peaks suggested no fluidization of epidermal lipids by lipid synthesis inhibitors. A direct correlation was observed between epidermal lipid synthesis inhibition, decrease in peak height or area, and percutaneous permeation of LD. Skin lipid synthesis inhibition by a combination of lipid synthesis inhibitors seems to offer a feasible approach for enhancing the transcutaneous delivery of LD.

KEYWORDS: skin lipids, lipid synthesis inhibitors, percutaneous permeation, attenuated total reflectance Fourier transform infrared spectroscopy, levodopa.

INTRODUCTION

Levodopa (LD) combined with a dopa-decarboxylase inhibitor is considered the gold standard of Parkinson's disease pharmacotherapy. LD exhibits a short half-life[1] of 1.4 hours, necessitating frequent oral administration. Oral administration of LD is associated with variable and unreliable clinical response because of its erratic oral absorption and first-pass metabolism. Further, the patients of Parkinson's disease are often geriatric and tend to have dementia and dysphasia. These types of patients cannot be expected to comply with oral administration. In addition, chronic oral administration results in altered absorption of LD owing to altered gastrointestinal tract (GIT) motility or competition with dietary amino acids for L-neutral amino acid transport systems.[2] This factor leads to "wear-off" of a dose of LD before the next dose, and the patients fluctuate between "on" and "off" responses.[3] Hence, strict maintenance of constant plasma LD concentration is required to prevent loss of effect in Parkinson's patients.

Transdermal delivery of LD is expected to offer continuous drug input over an extended period resulting in excellent patient compliance with more LD reaching the brain owing to elimination of presystemic first-pass. However, due to a very low log $K_{o/w}$ of −4.7, LD exhibits low percutaneous permeation.[4] This is further complicated by the fact that stratum corneum (SC) is a formidable barrier for permeation of polar drug molecules. Hence, it becomes imperative to search an effective means for enhancing transdermal delivery of LD in order to improve its pharmacokinetic response.

The intercellular domains of SC, which mediate the epidermal permeability barrier, are enriched in cholesterol, fatty acids, and ceramide.[5] Topical application of solvents is known to remove the lipids from SC. When the barrier is acutely perturbed by removal of these lipids, a sequence of biological response is initiated that includes accelerated synthesis of epidermal cholesterol, fatty acids, and ceramide.[6-8] This response replenishes SC lipid content leading to restoration of barrier function. The rate-limiting steps in the synthesis of cholesterol, fatty acids (as triglycerides), and sphingosine (a precursor for ceramide) can be inhibited by atorvastatin,[9] cerulenin,[10] and β-chloroalanine,[11] respectively. Inhibition of respective lipid synthesis is accompanied with the appearance of abnormal lamellar bodies and extracellular membrane structure leading to impaired barrier function of skin[12] and enhanced permeation of drugs.[13-17]

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Earlier investigations revealed that a single topical application of atorvastatin (AVN) (750 μg/7 cm^2), cerulenin (CN) (20 μg/7 cm^2), or β-chloroalanine (β-CA) (600 μg/7 cm^2) to ethanol-treated skin was able to inhibit the synthesis of cholesterol,[16] triglycerides,[17] and sphingosine,[16] respectively, up to 48 hours, thus maintaining the respective lipid content as low as that in freshly ethanol-treated skin. However, no significant increase ($P < .05$) in respective lipid synthesis inhibition was observed by higher doses of AVN (1000 μg/7 cm^2), CN (30 μg/7 cm^2), or β-CA (1200 μg/7 cm^2). Hence, the present investigation is designed to study the influence of topical application of a combination of optimized doses of AVN, CN, and β-CA to ethanol-treated viable rat skin on cholesterol, triglycerides, or sphingosine synthesis inhibition and on the percutaneous permeation of LD. In addition, attenuated total reflectance Fourier transform infrared spectroscopic (ATR-FTIR) studies were performed to assess the contribution of altered molecular conformations of skin lipids in enhancing the percutaneous permeation of LD.

Materials and Methods

Materials

LD and carbidopa were generous gift samples from Sun Pharma (Baroda, India). D-sphingosine, β-CA, and CN were purchased from Sigma Chemicals (St Louis, MO). AVN was a gift sample from Ranbaxy Research Laboratories (Gurgaon, India). Cholesterol and triglycerides estimation kits were purchased from Span Diagnostics (Surat, India) and Ranbaxy Laboratories (New Delhi, India), respectively. All other chemicals were of analytical reagent grade.

Albino Wistar rats of either sex (190-210 g), maintained on a standard laboratory diet and tap water ad libitum were used in this study. Various treatments were applied to the dorsal skin portion of rats 24 hours after shaving with an electric razor. Rats were killed after different time periods of skin treatment by excess inhalation of anesthetic ether, and treated skin portions were excised for further experiments. The protocol for this study was approved by the Institutional Animal Ethical Care Committee of the Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India.

Methods

Preparation of the Epidermis

The epidermis was prepared by soaking the freshly excised whole skin in water at 60°C for 45 seconds. The underlying dermis was removed by gentle scraping with spatula in order to obtain epidermal sheets.[18]

Attenuated total reflectance Fourier Transform Infrared Spectroscopic Analysis

ATR-FTIR spectra for both untreated epidermal sheets and those obtained from excised viable skin after treatment with AVN, CN, or β-CA were recorded in the frequency range of 3000 to 1000 cm^{-1} in absorbance mode. One hundred scans were made at a resolution of 2 cm^{-1} using deuteriated triglycine sulfate detector. Various doses of AVN (500, 750, or 1000 μg/7 cm^2), CN (10, 20, or 30 μg/7 cm^2), β-CA (400, 600, or 1200 μg/7 cm^2) or a combination of AVN (750 μg), CN (20 μg), and β-CA (600 μg) dissolved in 0.25 mL of propylene glycol:ethanol (7:3) mixture (PG-EtOH) was applied to patches prepared on dorsal skin surface of rats. Epidermal sheets were prepared from whole skin portions excised at different intervals of treatment. Samples of dry epidermal sheets were hydrated in a closed chamber containing a saturated solution of sodium chloride (75% relative humidity [RH] at 25°C). The samples were then subjected to ATR-FTIR analysis (410, Nicolet, Madison, WI). The epidermal samples were squeezed between KBr plates, clamped, and mounted for analysis. Attention was focused on characterizing the occurrence of peaks near 2850 and 2920 cm^{-1}, which are produced due to the symmetric and asymmetric C-H stretching absorbances, respectively. Area and height of these peaks were calculated using FTIR software. The percentage decrease in peak height or area was calculated by using the formula: 100 − [(absorbance peak height or area due to treatment ÷ absorbance peak height or area due to control) × 100]. Each ATR-FTIR experiment was performed in triplicate.

Influence of a Combination of AVN, CN and β-CA on Cholesterol, Fatty acids (as Triglycerides) and Sphingosine Content in Viable Rat Skin

Three patches (7 cm^2) were prepared on dorsal skin surface of rats by shaving with an electric razor. The treatment to these patches was given 24 hours after shaving. One patch was left untreated (control). The second patch was treated with ethanol (0.5 mL). The third patch was treated with ethanol followed by immediate application of a combination of AVN (750 μg), CN (20 μg), and β-CA (600 μg) solution prepared in 0.25 mL of PG-EtOH.[16,17] The animals were killed after 2, 8, 24, or 48 hours, and epidermis was separated from treated skin patches. These epidermal sheets were dried to constant weight, and total lipids were extracted by Folch method.[19] Cholesterol and triglycerides content in these extracts was determined by using respective diagnostic kit. Sphingosine content was determined spectrofluorometrically (LS 50B, Perkin Elmer, Buckinghamshire, UK) by using an excitation and emission wavelength of 340 and 455 nm, respectively, according to the method outlined by Sabbadini et al.[20] Percentage lipid

synthesis inhibited was calculated by using the formula: [{1 − (lipid content remaining in epidermis after inhibitor treatment ÷ lipid content in untreated epidermis)} × 100]. Three experiments were performed for each study.

In Vitro Permeation Studies of Levodopa

Two patches (7 cm^2), one on either side of spinal cord, were prepared by shaving with an electric razor. The treatment to these patches was given 24 hours after shaving. One patch was left untreated and served as control. The other patch received ethanol treatment followed by immediate application of a combination of AVN (750 μg), CN (20 μg), and β-CA (600 μg) dissolved in PG-EtOH (0.25 mL). Animals were killed after different intervals, and epidermal sheets obtained from treated portions were used for studying in vitro permeation of LD after stabilizing them for 4 hours in vertical Franz glass diffusion cells. LD (64 mg) dispersed in PG-EtOH mixture containing sodium sulfite (0.25% wt/vol) was loaded in the donor compartment. The receptor fluid consisted of phosphate buffer (pH 7.4) containing sodium azide (0.05% wt/vol), sodium sulfite (0.25% wt/vol), and polyethylene glycol (PEG) 400 (10% vol/vol). Samples (1 mL) were withdrawn at various time intervals throughout 48 hours and immediately analyzed spectrophotometrically (DU 640 B, Beckman, Fullerton, CA) for the amount of LD permeated at 280 nm.[21] A standard plot of LD (5-50 μg/mL) was prepared in phosphate buffer (pH 7.4) containing sodium sulfite (0.25% wt/vol) at 280 nm. Cartesian plots of cumulative amount of drug permeated into receptor compartment versus time were plotted for 3 experiments and flux (μg/cm^2/h) was calculated from the slope of steady-state portion of these plots. All experiments were performed in triplicate.

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/mL) dispersed in PG-EtOH was loaded into this cavity. Treatment given to various groups, each consisting of 5 rats can be summarized as follows: Group 1: normal skin + LD + carbidopa (16 mg); Group 2: ethanol perturbation + AVN (750 μg) +

Table 1. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy Attributes of Rat Epidermis Excised After Treatment With Atorvastatin for Different Time Periods*

	C−H Stretching of Rat Epidermal Lipid							
	Asymmetric				Symmetric			
Treatments	Peak Height	% Decrease	Peak Area	% Decrease	Peak Height	% Decrease	Peak Area	% Decrease
Untreated	3.76 ± 0.34	–	21.77 ± 2.78	–	3.14 ± 0.23	–	39.13 ± 2.14	–
PG-EtOH†	3.49 ± 0.31	7.10	19.94 ± 1.69	8.41	2.97 ± 0.25	5.32	37.56 ± 2.07	4.01
AVN (500 μg)								
2 hr	2.40 ± 0.15	36.17	14.86 ± 1.75	31.72	2.04 ± 0.11	34.84	16.12 ± 1.38	58.79
6 hr	2.48 ± 0.13	33.96	15.48 ± 1.39	28.89	2.19 ± 0.22	30.06	18.63 ± 1.24	52.29
8 hr	2.43 ± 0.11	35.21	16.93 ± 1.11	22.23	2.22 ± 0.19	29.10	21.11 ± 1.37	46.05
12 hr	2.52 ± 0.12	32.97	16.95 ± 1.25	22.14	2.30 ± 0.16	26.75	22.36 ± 1.24	42.85
24 hr	2.56 ± 0.16	31.91	18.91 ± 1.84	13.13	2.41 ± 0.19	23.15	28.30 ± 2.05	27.67
48 hr	2.59 ± 0.11	31.04	19.78 ± 1.26	9.11	2.55 ± 0.28	18.79	32.81 ± 1.49	16.15
AVN (750 μg)								
2 hr	2.39 ± 0.17	36.35	12.74 ± 0.95	41.48	1.84 ± 0.25	41.21	15.99 ± 1.31	59.13
6 hr	2.44 ± 0.21	27.04	11.54 ± 1.26	46.99	1.91 ± 0.17	38.98	17.59 ± 0.82	55.04
8 hr	2.66 ± 0.19	29.09	13.64 ± 1.06	37.34	2.14 ± 0.24	31.84	19.73 ± 1.58	49.58
12 hr	2.57 ± 0.25	35.02	15.19 ± 1.65	30.22	2.27 ± 0.16	27.61	20.93 ± 1.42	46.50
24 hr	2.74 ± 0.18	23.59	16.27 ± 1.15	25.26	2.38 ± 0.24	23.88	22.97 ± 1.29	41.28
48 hr	2.87 ± 0.23	31.57	16.64 ± 1.09	23.55	2.52 ± 0.28	19.74	26.25 ± 1.81	32.90
AVN (1000 μg)								
2 hr	2.29 ± 0.22	39.09	11.47 ± 0.89	47.42	1.71 ± 0.18	45.54	13.53 ± 1.18	65.87
6 hr	2.36 ± 0.17	37.07	10.48 ± 0.91	51.86	1.82 ± 0.20	41.84	15.56 ± 1.10	60.23
8 hr	2.44 ± 0.17	35.10	13.20 ± 0.74	39.34	2.05 ± 0.29	34.52	18.23 ± 0.87	53.40
12 hr	2.56 ± 0.23	31.95	14.57 ± 1.13	33.04	2.23 ± 0.26	28.80	19.88 ± 0.74	49.19
24 hr	2.59 ± 0.21	31.03	15.51 ± 1.17	28.73	2.31 ± 0.23	26.33	21.33 ± 1.21	45.47
48 hr	2.98 ± 0.27	20.58	15.83 ± 0.67	27.28	2.55 ± 0.28	18.79	25.30 ± 1.52	35.34

*PG-EtOH indicates propylene glycol-ethanol; and AVN, atorvastatin.

†Excised and treated for 48 hours.

Table 2. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy Attributes of Rat Epidermis Excised After Treatment With Cerulenin for Different Time Periods*

	C-H Stretching of Rat Epidermal Lipids							
	Asymmetric				Symmetric			
Treatments	Peak Height	% Decrease	Peak Area	% Decrease	Peak Height	% Decrease	Peak Area	% Decrease
Untreated	3.76 ± 0.34		21.77 ± 2.78		3.14 ± 0.23		39.13 ± 2.14	
PG-EtOH†	3.49 ± 0.31	7.10	19.94 ± 1.69	8.41	2.97 ± 0.25	5.32	37.56 ± 2.07	4.01
CN (10 μg)								
2 hours	3.03 ± 0.17	19.25	15.26 ± 1.82	29.87	2.23 ± 0.10	28.79	21.94 ± 1.71	43.93
6 hours	3.10 ± 0.22	17.39	16.88 ± 0.72	22.45	2.44 ± 0.13	22.10	24.49 ± 1.99	37.41
8 hours	3.16 ± 0.19	15.79	17.69 ± 1.65	18.71	2.52 ± 0.10	19.55	25.48 ± 2.27	34.88
12 hours	3.22 ± 0.13	14.28	18.74 ± 1.29	13.90	2.63 ± 0.13	16.15	28.05 ± 2.28	28.31
24 hours	3.29 ± 0.17	12.42	18.91 ± 1.85	13.12	2.66 ± 0.10	15.19	31.17 ± 2.54	20.34
48 hours	3.42 ± 0.24	8.88	19.51 ± 1.23	10.35	2.96 ± 0.11	5.64	35.54 ± 2.75	9.16
CN (20 μg)								
2 hours	2.97 ± 0.21	21.01	13.86 ± 1.44	36.32	2.05 ± 0.17	34.52	21.04 ± 1.45	46.21
6 hours	2.78 ± 0.23	25.98	14.78 ± 1.16	32.08	2.17 ± 0.17	30.79	22.45 ± 1.98	42.61
8 hours	2.65 ± 0.26	29.52	15.60 ± 0.82	28.34	2.32 ± 0.24	26.02	23.13 ± 1.13	40.88
12 hours	3.06 ± 0.26	18.53	16.13 ± 1.51	25.90	2.43 ± 0.20	22.51	26.02 ± 1.73	33.50
24 hours	3.18 ± 0.27	15.42	16.81 ± 1.18	22.78	2.52 ± 0.22	19.74	28.91 ± 1.68	26.11
48 hours	3.02 ± 0.22	19.52	18.45 ± 0.93	15.25	2.61 ± 0.21	16.88	32.13 ± 1.75	17.88
CN (30 μg)								
2 hours	2.57 ± 0.21	31.49	12.25 ± 1.36	43.73	1.98 ± 0.18	36.85	20.13 ± 1.40	48.55
6 hours	2.61 ± 0.12	30.42	14.06 ± 1.69	35.38	2.07 ± 0.09	33.98	21.44 ± 1.94	45.19
8 hours	2.74 ± 0.08	27.12	15.29 ± 1.14	29.76	2.29 ± 0.07	26.88	22.12 ± 1.46	43.46
12 hours	2.87 ± 0.10	23.59	15.58 ± 1.11	28.43	2.36 ± 0.09	24.84	25.64 ± 1.18	34.74
24 hours	3.07 ± 0.12	18.27	15.73 ± 1.23	27.72	2.44 ± 0.08	22.29	28.50 ± 0.92	27.15
48 hours	3.14 ± 0.19	16.41	17.91 ± 1.21	17.72	2.57 ± 0.10	18.15	31.15 ± 1.80	20.39

*CN indicates cerulenin; and PG-EtOH, propylene glycol-ethanol.

†Excised and treated for 48 hours.

LD + carbidopa; Group 3: ethanol perturbation + CN (20 μg) + LD + carbidopa; Group 4: ethanol perturbation + β-CA (600 μg) + LD + carbidopa; Group 5: ethanol perturbation + AVN (750 μg) + β-CA (600 μg) + CN (20 μg) + LD + carbidopa.

Blood samples (0.25 mL) were withdrawn from tail veins of rats after specified time intervals and centrifuged at 2500 rpm for 10 minutes at 4°C. The plasma was analyzed or intact LD by the method reported by Cotler et al.[22] Briefly, the deproteinized plasma (0.2 mL) was added to 1.0 mL of edetic acid solution (20 mg/mL). Water (1.0 mL) and 2 drops of ethanolic solution of naphthoresorcinol (2% wt/vol) were added, and the specimen was mixed. Subsequently, 5N sodium hydroxide (0.1 mL) was added, and the mixture was allowed to stand at room temperature for 5 minutes. The aqueous layer was washed once with 1-butanol (1.0 mL). The aqueous layer (1.0 mL) was saturated with sodium chloride (0.5 g) and then acidified with 6N hydrochloric acid (0.1 mL). The LD derivative was finally extracted with ethyl hexanol (2.0 mL) and analyzed spectrofluorometrically using excitation and emission wavelengths of 440 nm at 470 nm, respectively. The standard plot of LD (0.5-5.0 μg/mL) was prepared by dissolving it in plasma obtained from pooled rat blood samples.

Statistical Analysis

Statistical comparisons of all the data generated were made using the Student *t* test. The level of significance was taken as $P < .05$.

Results and Discussion

A dose- and time-dependent inhibition of skin lipid synthesis was evident in the ATR-FTIR spectra of epidermis treated with any lipid synthesis inhibitor. This lipid synthesis inhibition resulted in decrease in peak height and area for asymmetric and symmetric C-H stretching absorbances. Maximum decrease in peak area for asymmetric and symmetric C-H stretching absorbances was observed at 2 hours, 2 hours, and 8 hours after topical application of any dose of AVN (Table 1), CN (Table 2), and β-CA (Table 3), respectively. A dose of 750, 20, and 600 μg/7 cm^2 was found to be optimum for respective lipid synthesis inhibition because higher doses did not significantly decrease the peak

Table 3. ATR-FTIR Attributes of Rat Epidermis Excised After Treatment With β-CA for Different Time Periods*

Treatments	C−H Stretching of Rat Epidermal Lipids							
	Asymmetric				Symmetric			
	Peak Height	% Decrease	Peak Area	% Decrease	Peak Height	% Decrease	Peak Area	% Decrease
Untreated	3.76 ± 0.34	–	21.77 ± 2.78	–	3.14 ± 0.23	–	39.13 ± 2.14	–
PG-EtOH†	3.49 ± 0.31	7.10	19.94 ± 1.69	8.41	2.97 ± 0.25	5.32	37.56 ± 2.07	4.01
β-CA (400 μg)								
2 hours	3.26 ± 0.12	13.29	20.14 ± 1.82	7.47	2.59 ± 0.14	17.32	33.48 ± 2.19	14.44
6 hours	2.84 ± 0.30	24.31	19.27 ± 0.97	11.47	2.39 ± 0.23	23.69	26.22 ± 1.32	32.97
8 hours	3.19 ± 0.26	15.16	17.59 ± 0.73	19.17	2.02 ± 0.20	35.47	16.81 ± 1.39	57.04
12 hours	3.15 ± 0.25	16.14	19.45 ± 1.36	10.67	2.07 ± 0.19	33.98	29.75 ± 1.12	23.95
24 hours	2.86 ± 0.29	23.77	20.08 ± 0.83	7.76	2.17 ± 0.18	30.70	30.81 ± 1.51	21.24
48 hours	3.23 ± 0.28	14.09	21.58 ± 0.96	0.87	2.20 ± 0.27	29.93	33.91 ± 1.06	13.33
β-CA (600 μg)								
2 hours	3.33 ± 0.26	11.35	19.70 ± 0.91	9.50	2.43 ± 0.24	22.61	32.73 ± 2.53	16.35
6 hours	3.37 ± 0.30	10.37	18.75 ± 1.26	13.87	2.22 ± 0.23	29.29	21.70 ± 1.87	44.54
8 hours	3.12 ± 0.24	16.94	15.35 ± 1.37	29.49	2.03 ± 0.19	35.35	15.84 ± 1.20	59.51
12 hours	3.27 ± 0.34	13.03	17.09 ± 1.21	21.48	2.11 ± 0.28	32.70	27.62 ± 1.45	29.41
24 hours	3.34 ± 0.29	11.09	17.69 ± 1.46	17.69	2.24 ± 0.13	28.99	31.08 ± 1.78	20.57
48 hours	3.39 ± 0.25	9.84	19.03 ± 1.54	19.03	2.41 ± 0.21	23.24	31.78 ± 2.42	18.77
β-CA (1200 μg)								
2 hours	3.17 ± 0.30	15.69	19.26 ± 2.01	11.51	2.45 ± 0.09	21.97	30.70 ± 2.10	21.54
6 hours	2.93 ± 0.27	21.91	17.73 ± 1.96	18.53	2.16 ± 0.18	31.02	18.72 ± 1.70	52.15
8 hours	2.69 ± 0.23	28.45	13.16 ± 1.52	39.55	2.20 ± 0.13	29.94	15.06 ± 1.23	61.49
12 hours	2.95 ± 0.30	21.38	15.88 ± 1.38	27.03	2.12 ± 0.18	32.29	25.89 ± 1.93	33.84
24 hours	3.11 ± 0.28	17.21	16.56 ± 1.35	23.90	2.37 ± 0.24	24.33	29.24 ± 1.87	25.27
48 hours	3.30 ± 0.27	12.23	18.38 ± 1.19	15.56	2.32 ± 0.16	26.11	29.70 ± 1.80	24.09

*β-CA indicates β-chloroalanine; and PG-EtOH, propylene glycol-ethanol.

†Excised and treated for 48 hours.

area for either asymmetric or symmetric C-H stretching absorbances ($P < 0.05$). Reduction in the content of respective epidermal lipid can be directly correlated with the decrease in peak area for asymmetric and symmetric C-H stretching absorbances (Figure 1).

Figure 2 depicts ATR-FTIR spectra (3000-2750 cm^{-1}) of rat epidermis excised after different time intervals of treatment with 750 μg of AVN. Two prominent peaks near 2921 and 2850 cm^{-1} generally attributed to asymmetric and symmetric C-H stretching absorbances, respectively, of epidermal lipids were observed in all the spectra. Treatment of rat epidermis with PG-EtOH alone decreased the peak height and area of both asymmetric and symmetric C-H stretchings. However, the peak height decreased by only 7.1% and 5.32%, while peak area decreased by 8.41% and 4.01% for asymmetric and symmetric C-H stretching absorbances, respectively, in comparison with the untreated epidermis. Hence, while PG-EtOH dissolved lipid synthesis inhibitors and levodopa, it did not perturb the skin lipids appreciably. On the other hand, lipid synthesis inhibitors solubilized in PG-EtOH produced greater decrease in peak height and area for C−H stretching absorbances as compared with the effect of PG-EtOH alone. It is important to note that increasing the concentration of ethanol beyond 30% vol/vol in PG-EtOH mixtures is reported to drastically perturb skin lipids.[18] Therefore, a 7:3 mixture of PG-EOH was used as a vehicle in the present investigation in order to discern the effect of lipid synthesis inhibitors on lipid perturbation and percutaneous permeation of levodopa.

The height and area of asymmetric and symmetric C-H stretching bands have been found to be proportional to the amount of the lipids present in the epidermis. Therefore, a decrease in peak height or area can be attributed to epidermal lipid extraction.[18] Some enhancers that fluidize the epidermal lipids are reported to either increase the peak width alone or shift the C-H stretching peaks to higher wave number along with an increase in peak width. The shifting of a peak to higher wave number along with an increase in peak width is ascribed to the conversion of trans-conformers to gauche-conformers along the acyl chains. Therefore, the observed decrease in peak height and area for C-H stretching absorbances without any shift in their wave number indicates that PG-EtOH alone or its combination with any lipid synthesis inhibitor produced a predominant action akin to lipid extraction but did not fluidize the lipids.

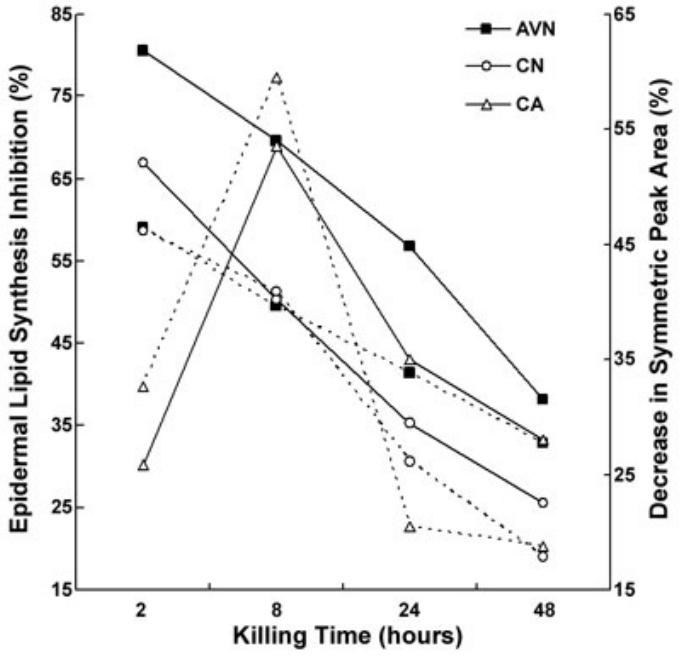

Figure 1. The inhibition of cholesterol, triglycerides, or sphingosine and concomitant decrease in symmetric C-H stretching area after treatment of viable rat epidermis with AVN (750 μg), CN (20 μg), or β-CA (600 μg), respectively. Solid lines indicate percentage epidermal lipid synthesis inhibition and broken lines indicate concomitant decrease in symmetric peak area.

It is worthy to note that treatment of viable rat epidermis with a combination of optimized doses of AVN, CN, β-CA produced significantly greater ($P < .05$) decrease in peak height and area of both asymmetric and symmetric C-H stretching absorbances as compared with their per se effect (Table 4). This finding seems to be due to simultaneous inhibition of all the 3 major epidermal lipids resulting in higher intensity of lipid synthesis inhibition after treatment with a combination of these inhibitors.

The effect of individual inhibitor was not discernable in the spectra. In order to discern the effect of the 3 inhibitors when applied in combination, the treated viable epidermis was excised and analyzed for cholesterol, triglycerides, and sphingosine content. The maximum synthesis inhibition of cholesterol, triglycerides, and sphingosine was found to be 82%, 67%, and 53%, respectively (Figure 3). These values were not significantly different ($P < .05$) from the maximum values obtained after treatment with the same dose of respective inhibitor when applied individually.[16,17] However, the in vitro permeation of LD across rat epidermis excised after any time interval of treatment with the combination of AVN, CN, and β-CA was found to be significantly higher ($P < .05$) than that obtained across epidermis excised after treatment with individual inhibitor (Figure 4). The in vitro permeation of LD across epidermis excised after 48 hours of single application of a combination of optimized doses of lipid synthesis inhibitors was still 3-fold greater as compared with that across normal epidermis. This result seems to be owing to synthesis inhibition of cholesterol, triglycerides, and sphingosine simultaneously at each time point after treatment with combination of inhibitors. Together, these results indicate relatively greater effect of treatment with combination of lipid synthesis inhibitors in influencing permeation of LD as compared with treatment with individual lipid synthesis inhibitor. It is important to note that the time of excision of skin through which maximum in vitro permeation of LD was observed coincided with the time point at which maximum lipid synthesis inhibition occurred. In addition, lipid synthesis inhibition produced a maximum decrease in peak areas for asymmetric and symmetric C-H stretching absorbances at the same time point (Figure 1). This finding suggests a direct correlation between the magnitudes of epidermal lipid synthesis inhibited or decrease in peak area for asymmetric and symmetric C-H stretching absorbances and enhanced permeation of LD.

The pharmacokinetic studies on systemic delivery of percutaneously applied LD were conducted in Wistar rats

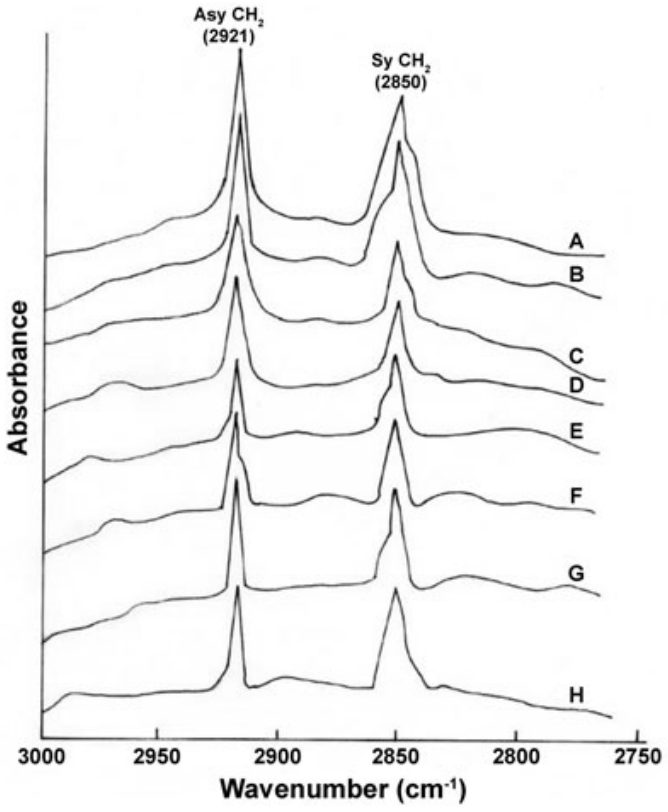

Figure 2. ATR-FTIR spectra of viable rat epidermis: (A) untreated; (B) excised and treated with PG:EtOH for 48 hours; (C) treated with ethanol + atorvastatin (750 μg) and excised after 2 hours; (D) 6 hours; (E) 8 hours; (F) 12 hours; (G) 24 hours; (H) 48 hours. All dried epidermal sheets were hydrated at 75% RH prior to ATR-FTIR analysis. Experiments were performed in triplicate.

Table 4. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy Attributes of Rat Epidermis Excised After Treatment With a Combination of AVN (750 μg), CN (20 μg), and β-CA (600 μg) for Different Time Periods*

	C-H Stretching of Rat Epidermal Lipids							
	Asymmetric				Symmetric			
Treatments	Peak Height	% Decrease	Peak Area	% Decrease	Peak Height	% Decrease	Peak Area	% Decrease
Untreated	3.76 ± 0.34		21.77 ± 2.78		3.14 ± 0.23		39.13 ± 2.14	
PG-EtOH†	3.49 ± 0.31	7.10	19.94 ± 1.69	8.41	2.97 ± 0.25	5.32	37.56 ± 2.07	4.01
2 hours	1.96 ± 0.16	47.87	9.50 ± 0.77	56.33	1.32 ± 0.12	57.86	13.32 ± 0.70	65.95
6 hours	2.13 ± 0.24	43.19	10.59 ± 1.09	51.34	1.53 ± 0.14	51.17	15.65 ± 1.25	60.00
8 hours	1.88 ± 0.12	50.00	8.28 ± 0.75	61.96	1.38 ± 0.07	56.05	10.70 ± 0.74	72.65
12 hours	2.30 ± 0.11	38.67	11.75 ± 1.14	46.02	1.63 ± 0.15	47.99	16.82 ± 1.16	57.01
24 hours	2.41 ± 0.09	35.82	12.42 ± 0.71	42.92	1.85 ± 0.13	41.08	20.34 ± 1.19	48.02
48 hours	2.71 ± 0.15	28.19	14.14 ± 0.76	35.03	2.13 ± 0.14	32.16	23.09 ± 0.98	40.99

*AVN indicates atorvastatin; CN, cerulenin; β-CA, β-chloroalanine; and PG-EtOH, propylene glycol-ethanol.
†Excised and treated for 48 hours.

following various treatments (Figure 5). LD was not detectable in plasma when it was applied without carbidopa to untreated skin of rats. This was probably owing to the degradation of LD by dopa decarboxylase. After coapplication of carbidopa (dopa decarboxylase inhibitor), a very small amount of LD permeated into the systemic circulation (Group 1), indicating significant resistance offered by intact skin for permeation of polar drug molecules. An increase in the systemic delivery of LD was observed when LD-carbidopa combination was applied to ethanol-treated skin. But, the effective concentration (C_{eff}) of LD was not achieved and the plasma level sharply declined to negligible concentration by 12 hours. This result could be attributed to the resistance put forth by lipids whose synthesis is reported to get accelerated in a bid to normalize their content following ethanol-perturbation.[7,8,16,17] It is noteworthy that significantly higher ($P < .05$) plasma LD concentration was achieved when the transdermal formulation contained AVN (750 μg), CN (20 μg), β-CA (600 μg) or a combination of these lipid synthesis inhibitors (Groups 2-5). C_{eff} of LD was achieved within 2, 4, or 6 hours after application of AVN, CN, or β-CA, respectively. Shorter time required for achieving C_{eff} after application of AVN or CN as compared with that after β-CA application seems to be due to implication of cholesterol and fatty acids[23] in the initial phase and to sphingosine synthesis[24] in the later phase of epidermal barrier recovery. Although the time required to achieve C_{eff} by application of β-CA was 6 hours, the plasma concentration was maintained above C_{eff} for

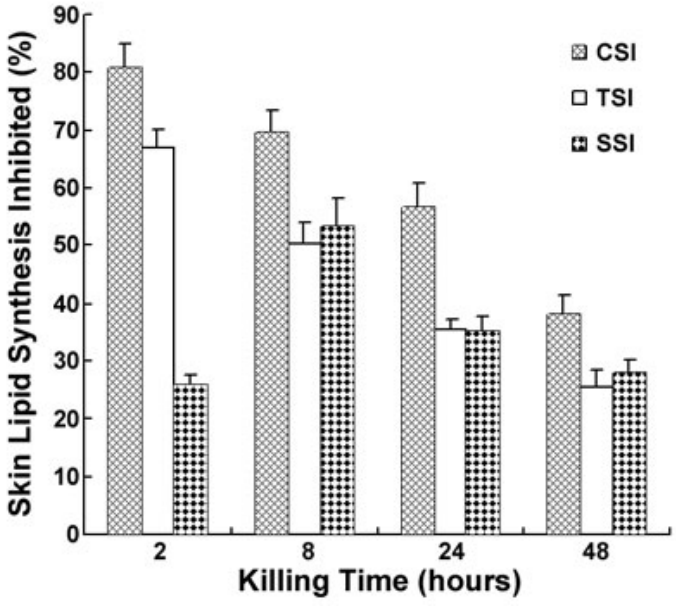

Figure 3. Cholesterol synthesis inhibited (CSI), triglycerides synthesis inhibited (TSI), and sphingosine synthesis inhibited (SSI) in viable rat epidermis treated with a combination of AVN (750 μg), CN (20 μg), and β-CA (600 μg) and excised after different time periods. Error bars indicate mean ± SD of 3 experiments.

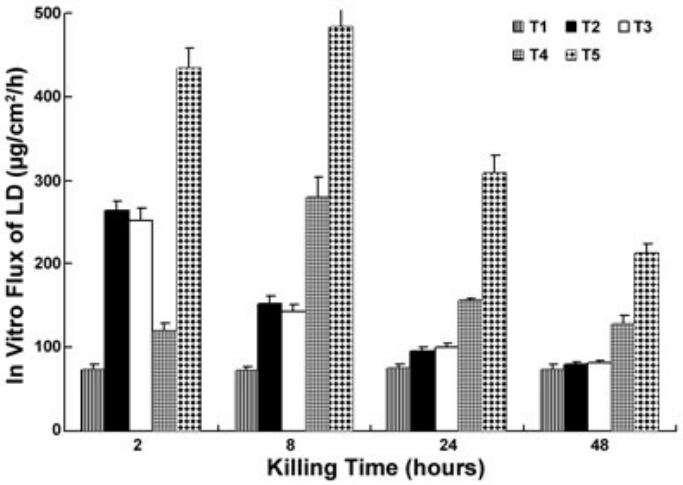

Figure 4. In vitro permeation of LD across rat epidermis excised after no treatment, T1; treatment with AVN (750 μg), T2; CN (20 μg), T3; β-CA (600 μg), T4; and their combination, T5. Error bars indicate mean ± SD of 3 experiments.

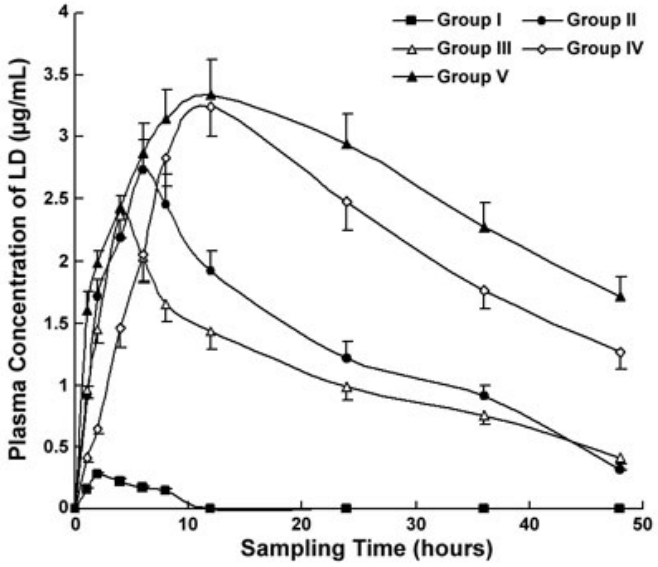

Figure 5. Percutaneous permeation of LD in rats after no treatment (Group 1); ethanol treatment followed by AVN (750 µg) treatment (Group 2); CN (20 µg) treatment (Group 3); β-CA (600 µg) treatment (Group 4); or a combination of the doses of AVN, CN. and β-CA (Group 5). Error bars indicate mean ± SD of 3 experiments.

36 hours as opposed to 12 hours and 8 hours after treatment of skin with either AVN or CN, respectively. However, when a combination of these lipid synthesis inhibitors was used, the time required for achieving effective plasma concentration reduced to 1 hour and C_{eff} was maintained over 48 hours.

CONCLUSIONS

A combination of ethanol, AVN, CN, and β-CA appear to be a good choice for inhibiting epidermal lipid synthesis. The ATR-FTIR spectra of lipid synthesis inhibitor(s)-treated epidermis exhibited significant ($P < .05$) decrease in peak height and area of both asymmetric and symmetric C-H stretching absorbances suggesting lipid extraction. The percutaneous delivery of LD in rats after single application of a transdermal patch containing combination of lipid synthesis inhibitors revealed reduction in the time to achieve plasma C_{eff} to 1 hour and the C_{eff} was maintained through 48 hours.

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