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ORIGINAL ARTICLE

Transdermal delivery of carvedilol in rats: probing the percutaneous permeation enhancement mechanism of soybean extract–chitosan mixture

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Abstract

Background: This study was designed for investigating the effect of soybean (SS) extract and chitosan (CTN) in facilitating the permeation of carvedilol (CDL) across rat epidermis. *Method*: Transdermal flux of carvedilol through heat-separated rat epidermis was investigated in vitro using vertical Keshary–Chien diffusion cells. Biophysical and microscopic manifestations of epidermis treated with SS-extract, CTN, and SS extract–CTN mixture were investigated by using DSC, TEWL, SEM, and TEM. Biochemical estimations of cholesterol, sphingosine, and triglycerides were carried out for treated excised as well as viable rat epidermis. The antihypertensive activity of the patches in comparison to that after oral administration of carvedilol was studied in deoxycorticosterone acetate-induced hypertensive rats. *Results*: The solubility of CDL was found to be maximum in the presence of 1% (w/v) SS extract. The $K_{IPM/PB}$ of CDL decreased with increase in concentration of SS extract. The in vitro permeation of CDL across rat epidermis increased and was maximum with combination of SS extract and chitosan (CTN). Biochemical and microscopic studies revealed the initiation of reversal of barrier integrity after 12 hours. Furthermore, the application of patches containing SS extract–CTN mixture resulted in sustained release of carvedilol, which was able to control the hypertension in deoxycorticosterone acetate (DOCA) induced hypertensive rats through 24 hours. CTN was found to potentiate the permeation enhancing activity of SS extract. *Conclusion*: The developed transdermal patches of CDL containing SS extract–CTN mixture exhibited better performance as compared to oral administration in controlling hypertension in rats.

Key words: *Differential scanning calorimetry; pharmacokinetic; saponin; scanning electron microscopy; solubility; transmission electron microscopy*

Introduction

Innovations in the area of drug delivery are taking place at a much faster pace as compared to the last two decades. Improved patient compliance and effectiveness are inextricable aspects of a new drug delivery system. Transdermal delivery offers several biomedical advantages over conventional routes including avoidance of presystemic and systemic first pass metabolism, extending drug release for long duration besides providing a convenient noninvasive and easily terminable means for systemic as well as topical drug delivery.

The implacable surge in the arena of transdermal drug delivery has led to the consideration of many aspects of percutaneous penetration process. Several physical (sonophoresis, iontophoresis, electro-osmosis, electroporation, and temperature), chemical methods or formulations reported in the literature have been investigated for elevating the amount of drugs delivered across and into the skin. Currently, the most widely used approach for drug permeation enhancement across the stratum corneum (SC) is the use of chemical penetration enhancers (sorption promoters and accelerants). In the class of synthetic enhancers, recently used enhancers are Transkarbam, a synthetic surfactant[1], 6-dimethylaminohexanoic acid dodecyl ester[2], dodecyl 2-(dimethylamino) propionate[3], and a series of transdermal permeation enhancers based on amino acids[4] and dicarboxylic acid esters[5]. The modes of action of these agents have been reviewed in detail by several

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authors[6]. These enhancers are reported to exert multiple effects such as increasing the diffusivity of the drug in the skin, causing SC lipid fluidization, decreasing the barrier function (a reversible action), and enhancing the thermodynamic activity of the drug in the vehicle and skin. This results in a reservoir of drug within the skin, affecting the partition coefficient of the drug, which ultimately results in release of drug from the formulation into the upper layers of the skin[7]. Organic solvents, tape stripping, and surface active agents are known to remove skin lipids. However, these treatments are not safe[8].

Typically, a successful enhancer should be able to partition into and interact with skin constituents to induce a temporary and reversible increase in skin permeability[9]. Most notably, the mechanism by which drug molecules penetrate the dermal barrier and the physicochemical properties of the permeant are inviting greater attention. These issues are now more amenable to discussion because of the improved understanding of the SC structure.

There has been much debate over the past decade on the route of drug penetration. Experimental evidence suggests intercellular route to be predominantly followed under normal circumstances[10]. The diffusional path length is therefore much longer than the simple thickness of the SC (~20 μm) and has been estimated as long as 500 μm. Importantly, the intercellular spaces contain structured lipids, and a diffusing molecule has to cross a variety of lipophilic and hydrophilic domains before it reaches the junction between the SC and the viable epidermis[11].

Two primary considerations arise while using surfactants for enhancing drug transport across biological membranes. The first consideration relates to temporary alteration of integrity of the rate limiting barrier, which can be manifested in the form of increased membrane fluidity, solubilization and/or extraction of lipids present in biological membranes, and alterations in the tight junction properties[12,13]. The second consideration entails the interaction of drug with surfactants. These physicochemical interactions manifest themselves in terms of enhanced solubility and/or dissolution of drug, prevention of drug precipitation if administered in solution form, and reduction in drug activity. The increase/reduction in drug activity is a consequence of interaction of drug with aggregates and/or micelles formed by the surfactant[14].

Many of the synthetic enhancers cause permanent epidermal damage that can only be repaired by SC regeneration[15]. However, for other enhancers, the increased permeability of SC may return to its normal state after their removal from the site of application. This temporary effect is attributed to the transient interactions between the enhancers and the SC lipids, which is the major diffusion passage for almost all small chemicals[16]. Saponins are natural surfactants and hence possess great potential for use as percutaneous permeation enhancer. Soya (SS) extract contains triterpenoidal saponins. Chitosan (CTN) is a polysaccharide and carries high positive charge when dissolved in organic acids. It is known to open the tight junctions in skin[17] can be envisaged to disturb the ordering of skin lipids.

Carvedilol (CDL) possesses ideal characteristics of low molecular weight (406.5), favorable logarithmic partition coefficient [log octanol/water, 0.58 ± 0.02; log octanol/buffer (pH 7.4) 0.61 ± 0.06], smaller dose range (25–50 mg), short plasma half-life, and poor oral bioavailability (high hepatic metabolism) for transdermal formulation.

This investigation is aimed at studying the effect of SS extract and CTN on permeation of CDL and unveiling its influence on rat epidermal microstructure and microchemical constituents.

Materials and methods

Materials

CDL and CTN were received as gift samples from Ranbaxy Laboratories Ltd. (Gurgaon, India) and Central Institute of Fisheries (Matsyapuri, Cochin, India), respectively. Deoxycorticosterone acetate (DOCA), cholesterol (CHOL), and triglycerides (TGS) estimation kits were purchased from Sigma (Bangalore, India), Span Diagnostics (Surat, India), and Ranbaxy Laboratories (New Delhi, India), respectively. All other chemicals were of analytical reagent grade.

Methods

Extraction procedure

Soybean seed powder was defatted with hexane, and the soxhlet extraction was done using ethanol (75%, v/v), followed by evaporation of extract to dryness. The extract was then dissolved in *n*-butanol and shaken with water. The butanol layer was separated and evaporated to dryness. Thereafter, the residue was dissolved in alcohol, and the resulting solution was injected into ether with stirring. The precipitate was recovered by filtration to yield a mixture of saponins containing soyasapogenols[18].

Standardization of soybean extract

Standardization was done by thin layer chromatographic technique. Silica gel was used as the stationary phase and chloroform/methanol/water (65:35:10) was used as the mobile phase. Clear reddish violet spots appeared when a mixture of ceric sulfate (1%, v/v) and sulfuric acid (10%, v/v) was sprayed against the support under heat. R_f value was found to be 0.27. In addition, standardization by infrared absorption was carried out using KBr

pellets. Infrared absorption spectra of the extract revealed peaks at 3434, 2924, 1739, 1457, and 1045 cm^{-1}, which were in accordance with the reported values[18].

Preparation of epidermal sheets

Epidermal sheets were obtained from Albino Wistar rats of either sex (175–225 g) employing the procedure for separating epidermis from whole thickness skin as described by Kligman and Christophers[19]. Freshly separated epidermal sheets were used in all the experiments.

Determination of critical micelle concentration of SS, its effect on solubility and partition coefficient of CDL

SS extract (0.1–2%, w/v) was dissolved in a 7:3 mixture of propylene glycol (PG):ethanol (ETOH). Each solution was subjected to surface tension measurement for finding the critical micelle concentration (CMC) value. Influence of SS extract on solubility of CDL was investigated by adding excess CDL to phosphate buffer (PB, pH 7.4) containing PEG 400 (10%, v/v) and different concentrations of SS extract (0.1–2%, w/v). Excess CDL was added separately to PB solutions not containing SS extract. All solutions were stored at 37 ± 2°C in shaker incubator for 24 hours. The solutions were filtered through G-4 filters, and the filtrates were immediately analyzed for CDL spectrofluorometrically employing excitation and emission wavelength of, respectively, 282 and 350 nm.

The partition coefficient was determined by adding CDL (50 mg) to a 1:4 mixture of isopropyl myristate (IPM) and PB containing PEG 400 (10%, w/v). The mixture was stirred at 37 ± 2°C in shaker incubator for 24 hours. The concentration of CDL in PB was determined spectrofluorometrically employing excitation and emission wavelengths of 282 and 350 nm, respectively. The amount of CDL partitioned into IPM was calculated by difference. The ratio of the amount of CDL in IPM to that in PB was calculated. All experiments were performed in triplicate.

Influence of different donor formulations on in vitro permeation of CDL

Freshly obtained rat epidermal sheets were mounted on a vertical Keshary–Chien diffusion cell. CDL dispersed in 4 mL of PG:ETOH (7:3) mixture was mixed with different concentrations of SS extract (0.1–2%, w/v), CTN (0.5%, w/v), or solution containing mixture of SS extract (1%, w/v) and CTN (0.5%, w/v) and loaded in the donor compartment. The donor compartment was sealed using a parafilm. The receptor fluid was maintained at 32 ± 1°C and contained a mixture of PB, sodium azide (0.05%, w/v), and PEG 400 (10%, w/v). The receptor fluid samples were withdrawn repeatedly through 48 hours and analyzed spectrofluorometrically employing excitation and emission wavelengths of 282 and 350 nm, respectively. An equal volume of fresh receptor fluid was replenished in the receptor compartment after each sampling.

In vitro permeation of CDL across SS extract-treated viable rat epidermis

Two patches (~7 cm^2), one on either side of spinal cord, were prepared by shaving with an electric razor and left undisturbed for 24 hours. One patch was left untreated and served as control. The other patch received application of SS extract (1%, w/v), CTN (0.5%, w/v), or solution containing a mixture of SS extract (1%, w/v) and CTN (0.5%, w/v). The animals were killed after 4, 12, 24, or 48 hours of application. Epidermal sheets obtained from these excised patches were used for studying the in vitro permeation of CDL using vertical Keshary–Chien diffusion cells as described earlier.

Influence of different treatment on cholesterol, triglycerides, and sphingosine content in excised and viable rat epidermis

Freshly excised rat epidermal sheets were treated with SS extract (1%, w/v), CTN (0.5%, w/v), or SS extract–CTN mixture for 48 hours. Transdermal patches containing these formulations were prepared using adhesive tape, polyethylene backing membrane, and a rubber ring. They were applied to shaved skin on the dorsal portion of rats. The animals were killed after 4, 12, 24, or 48 hours, and the epidermis was separated from the whole skin patches. All epidermal sheets were dried to constant weight at 50°C, and total lipids were extracted by Folch method[20]. CHOL and TGS content in these extracts was determined by using respective diagnostic kit. Sphingosine (SGE) content was determined spectrofluorometrically (SL-174 spectrofluorometer; ELICO Limited, Hyderabad, India) using excitation and emission wavelengths of 340 and 455 nm, respectively, according to the method outlined by Sabbadini et al.[21]

Differential scanning calorimetric analysis

Differential scanning calorimetric (DSC) analysis (ambient to 120°C, 1°C/min) was carried out on excised untreated rat epidermal sheets as well as those obtained after treatment with different concentrations of SS extract (0.1–1%, w/v), CTN (0.5%, w/v), or SS extract–CTN mixture (Mettler Toledo Star System, 821 E; Mettler Toledo, Schwerzenbach, Switzerland). Epidermal sheets were washed with distilled water thoroughly and were dried. 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.

Scanning electron microscopy and transmission electron microscopy

Whole thickness skin samples were removed following 4, 12, 24, or 48 hours of application of SS extract (1%, w/v), CTN (0.5%, w/v), or SS extract–CTN mixture to viable rat skin and also after 48 hours treatment to excised skin. Biopsies (1 × 4 mm^2) were processed according to

the methods described by Singh et al.[22] and Van den Bergh et al.[23], respectively, for scanning electron microscopy (SEM) (LEO 435VP) and transmission electron microscopy (TEM) investigations (Morgani-268). These photomicrographs were compared with those of PB-treated skin samples.

Transepidermal water loss studies

Two patches (~7 cm^2), one on either side of spinal cord, were prepared by shaving with an electric razor and left undisturbed for 24 hours. One patch was left untreated and served as control. The other patch received application of SS extract (1%, w/v), CTN (0.5%, w/v), or solution containing mixture of SS extract and CTN. The control and treatment sites were marked as circular area (~7 cm^2) with a felt tip marker on the dorsal portion of the rat. The patches were removed after 4, 12, 24, or 48 hours of application. The rats were anaesthetized at the time of measuring the transepidermal water loss (TEWL). Measurements of TEWL were taken at 0, 1, 2, 4, 6, 12, 20, 24, 36, or 48 hours of application and after 48 hours of removal of the patch. The TEWL was measured using Tewameter TM 210 (Courage and Khazaka Electronic GmbH, Kohn, Germany). The probe of the Tewameter was placed perpendicular to the surface of the skin, and a stable reading of TEWL was reached in about 60 seconds. The results were expressed in g/m^2/h. The measurements were performed in an acclimatized room with a mean relative humidity of 50.2 ± 6.9% and a mean room temperature of 21.6 ± 0.6°C as described by Rogiers[24].

Efficacy of transdermal patches in DOCA-induced hypertensive rats

Male Wistar rats (150–160 g) of 6–8 weeks age were used in the study. They were fed with standard laboratory chow diet and given water ad libitum before initiation of the study and were exposed to 12 hours of light–dark cycle. The experimental protocol was approved by Institutional Animal Ethics Committee of the Punjabi University, Patiala, and the care and handling of the animals were in accordance with the National Institutes of Health guidelines.

Acclimatization and preparation of the animals was done by following the procedure described by Khazaei and Nematbaksh[25]. The blood pressure was measured by tail-cuff method using noninvasive blood pressure monitoring system (Model 229IITC; IITC Life Science, Woodland Hills, CA, USA). Hypertension was induced by administering DOCA (30 mg/kg, s.c.) twice weekly for 5 weeks.[18] The animals selected after 4 weeks of DOCA administration were divided into three groups (n = 6). Group I served as control, group II received CDL 10 mg/kg orally, group III received application of transdermal patch containing SS extract–CTN mixture. BP was measured at different time intervals (0, 1, 2, 4, 6, 8, 20, 22, 24, 30, 36, or 48 hours).

Evaluation of pharmacokinetic parameters of carvedilol in animals

Wistar albino rats (200–250 g) were used. A patch of ~7 cm^2 was prepared on the dorsal side by shaving with electric shaver and left untreated for 24 hours. The rats were divided into three groups (n = 6). Group I was administered CDL orally (10 mg/kg as a 0.5% carboxymethyl cellulose suspension), group II received transdermal patch containing CDL dispersed in PG:ETOH, and group III received transdermal patch containing CDL and SS extract–CTN mixture. The blood samples were withdrawn at different time intervals (1, 2, 4, 6, 8, 10, 12, 24, 28, 36, or 48 hours) and analyzed at 242 nm by HPLC (Waters, Veinna, Austria). The system comprised of Waters 600 controller pump, Waters inline degasser AF, Photodiode Array Detector-2996, C_8 5 µ spherisorb column (4.6 × 250 mm^2), and Empower Pro software. The mobile phase was a 40:60 mixture of pH 3 phosphate buffer (0.2 M) and acetonitrile. The flow rate was adjusted to 1 mL/min. Nimesulide was used as an internal standard. CDL was detected at 242 nm.

The protocol was approved by Animal ethics Committee of the Department of Pharmaceutical Sciences, Punjabi University, Patiala. The concentration of CDL in the plasma samples was determined, and pharmacokinetic parameters were estimated by using WinNonlin Version 5.2 (Pharsight Corp., Mountain View, CA, USA).

Statistical analysis

Analysis of variance (ANOVA) followed by Duncan or Dunnett test was used for statistical comparison of the data. Significance level was fixed at 0.05.

Results and discussion

Solubility and partition coefficient ($K_{IPM/PB}$)

The solubility of CDL was observed to increase 10.51-fold in PB in the presence of 1% (w/v) SS extract as compared to that in PB. The enhancement of aqueous solubility of CDL by SS extract (1%, w/v) resulted in a decrease in partition coefficient of CDL. However, further increase in concentration of SS extract reduced the aqueous solubility of CDL and increased its partition coefficient (Table 1). The enhancement of aqueous solubility of CDL by SS extract (1%, w/v) could be attributed to the surfactant nature of SS extract. The $K_{IPM/PB}$ of CDL decreased with increase in concentration of SS extract due to increase in its aqueous solubility. However, the aqueous solubility decreased sharply and was accompanied with increase in $K_{IPM/PB}$ when the concentration of SS extract was increased beyond 1% (w/v) (Table 1).

Table 1. The influence of saponins of soya (SS) on solubility and partition coefficient of carvedilol.

	Characteristic	
Concentration (%, w/v)	Solubility (μg/mL) (in PB along with PEG 400)	Partition coefficient ($K_{IPM/PB}$)
Nil[a]	0.383 ± 0.019	17.403 ± 0.871
Nil	1.538 ± 0.076	4.339 ± 0.217
0.1% SS	4.025 ± 0.201	1.656 ± 0.083
0.5% SS	5.252 ± 0.263	1.269 ± 0.063
0.75% SS	6.124 ± 0.306	1.089 ± 0.054
1% SS	6.998 ± 0.349	0.952 ± 0.047
1.5% SS	6.002 ± 0.300	1.111 ± 0.055
2% SS	5.891 ± 0.294	1.131 ± 0.056

IPM, isopropyl myristate; PB, phosphate buffer, pH 7.4 containing PEG 400 and sodium azide. [a]PB without PEG 400 (all the experiments were carried out in triplicate).

CMC

The surface tension of solutions decreased sharply when the concentration of SS extract was increased to 1% (w/v). However, further increase in SS extract concentration did not reduce the surface tension. This indicated 1% (w/v) to be the CMC of SS extract. The decrease in solubility and increase in partition coefficient seems to be due to the formation of micelles of SS extract at 1% (w/v).

In vitro permeation studies

The data summarized in Table 2 shows that the permeation of CDL using SS extract (1%, w/v), CTN (0.5%, w/v), or combination of SS extract (1%, w/v), and CTN (0.5%, w/v) as donor formulations across excised rat epidermis was significantly higher ($P < 0.05$) as compared to that using propylene glycol–ethanol (7:3) mixture. Maximum flux of CDL 118.83 (μg/cm^2/h) was observed when mixture of CTN and SS extract was used as donor vehicle.

Table 2. In vitro permeation of CDL from different formulations across excised rat epidermis.

Donor vehicle	Flux (μg/cm^2/h)	Enhancement ratio (ER)
PG:ETOH (7:3)	22.78 ± 2.34	—
Buffer (pH 6)	20.6 ± 1.81	—
SS extract (%, w/v)		
0.1%	22.9 ± 1.25	1.01
0.5%	37.33 ± 2.32	1.64
0.75%	40.9 ± 2.88	1.79
1%	51.11 ± 3.26	2.24
2%	38.13 ± 2.41	1.67
CTN (0.5%)	103.5 ± 5.19	4.54
CTN (0.5%, w/v) + SS (1%, w/v)	118.83 ± 6.22	5.21

PG, propylene glycol; ETOH, ethanol (all the experiments were carried out in triplicate).

Table 3. In vitro permeation of CDL across epidermis excised after treatment of viable rat epidermis.

Formulation	Excision time (hours)	Flux (μg/cm^2/h)	Enhancement ratio (ER)
SS (1%, w/v)	4	22 ± 1.76	0.96
	12	41.2 ± 2.99	1.81
	24	23.4 ± 1.24	1.03
	48	20.1 ± 1.68	0.88
CTN (0.5%, w/v)	4	24.94 ± 1.05	1.09
	12	60.13 ± 3.68	2.63
	24	33.28 ± 2.82	1.45
	48	30.29 ± 2.91	1.32
SS (1%, w/v) + CTN (0.5%, w/v)	4	28.56 ± 0.98	1.25
	12	75.68 ± 1.24	3.32
	24	53.51 ± 2.39	2.34
	48	32.24 ± 2.77	1.41

All the experiments were carried out in triplicate.

Similarly, the permeation of CDL across epidermis excised from viable skin after treatment for 12 hours with a mixture of SS and CTN was observed to be significantly greater ($P < 0.05$) as compared to that obtained across epidermis treated with either SS extract or CTN alone (Table 3). Further, comparison of Tables 2 and 3 indicates significantly greater ($P < 0.05$) permeation of CDL across excised epidermis as compared to that across epidermis excised from viable skin after any treatment. The in vitro permeation of CDL across rat epidermis increased 2.24-fold in the presence of 1% (w/v) concentration of SS extract. However, no improvement in CDL permeation was observed on further increasing the concentration of SS extract in the donor formulation (Table 2). These observations are in agreement with the report of Nokhodchi et al.[26] who found 10-fold enhancement of in vitro permeation of diclofenac in presence of 0.1% (w/v) glycyrrhizin (GHN) to reduce to 1.72-fold in presence of higher (0.5%, w/v) concentration of GHN. Further, negligible permeation of diazepam in the presence of concentrations of Tween 80 greater than its CMC has been reported by Shokri et al.[27] Similar findings have been reported by other workers[28]. Hence, availability of less quantity of CDL in free form due to its entrapment in micelle seems to be responsible for the observed low permeation of CDL in presence of SS extract concentrations greater than its CMC of 1% (w/v).

Drug permeability across skin is a function of its initial partitioning into the SC, diffusion across the SC, partitioning out of the SC into the epidermis, and finally, diffusion across the epidermis. Therefore, results of solubility, $K_{IPM/PB}$, and surface tension measurements together implicate formation of micelles in reducing the in vitro permeation of CDL when SS extract was present in concentrations greater than its CMC.

CTN is reported to open the epidermal tight junctions. The effect of CTN on CaCo-2 monolayer tight junction

integrity for anti-goat HRP secondary antibodies was found to be maximum at concentration of 0.5% and translocation of proteins Zo-1 and occluding was completed in 48 hours[17]. Therefore, it was hypothesized that treatment of viable rat skin with a combination of SS extract and CTN could lead to potentiation of CDL permeation. The in vitro permeation of CDL in the presence of mixture of SS extract (1%, w/v) and CTN (0.5%, w/v) was further enhanced to 5.2-fold (Table 2).

Table 3 indicates that the in vitro permeation of CDL across epidermis excised from viable skin after 12 hours treatment with any formulation was greater than that across epidermis excised at other time periods. However, comparison of this data with that presented in Table 2 indicates significantly less ($P < 0.05$) in vitro permeation of CDL across epidermis excised from viable skin treated with different formulations as compared to that across excised epidermis using same formulations. This suggests that SS extract, CTN, or mixture of SS extract and CTN was less effective in promoting the passage of CDL when applied to viable skin. This could be attributed to the steady recovery of the microconstituents in viable skin after perturbation due to their continuous synthesis in an attempt to restore the original status and function of skin.

Table 4. Effect of treatment of soya (SS) extract, chitosan (CTN), or their mixture on lipids of epidermis in excised and viable rat skin.

	Skin lipids extracted (%)		
Formulation	Cholesterol	Triglyceride	Sphingosine
Skin type: excised			
SS (%, w/v)			
0.1	7.99 ± 1.22	7.03 ± 1.51	3.22 ± 0.49
0.5	11.72 ± 1.97	9.11 ± 2.06	7.20 ± 0.85
0.75	15.91 ± 2.10	10.77 ± 2.11	8.01 ± 0.48
1	18.57 ± 2.09	11.40 ± 2.33	8.44 ± 0.84
2	20.99 ± 1.24	13.33 ± 2.49	8.69 ± 1.08
CTN (0.5%, w/v)	19.12 ± 2.17	6.02 ± 0.58	7.35 ± 0.58
SS (1%, w/v) + CTN (0.5%, w/v)	23.66 ± 4.87	17.05 ± 3.41	12.09 ± 2.09
Skin type: viable			
SS (1%, w/v)			
4 hours	6.08 ± 0.39	5.02 ± 1.28	0.69 ± 0.11
12 hours	10.04 ± 1.08	11.35 ± 2.43	1.99 ± 0.67
24 hours	5.13 ± 0.84	3.80 ± 0.58	0.78 ± 0.18
48 hours	1.89 ± 0.72	2.00 ± 0.64	0.50 ± 0.42
CTN (0.5%, w/v)			
4 hours	1.71 ± 0.98	1.91 ± 0.25	0.69 ± 0.27
12 hours	7.10 ± 1.11	12.01 ± 2.01	2.40 ± 0.08
24 hours	2.53 ± 0.99	0.51 ± 0.11	0.29 ± 0.06
48 hours	2.01 ± 0.84	0.49 ± 0.88	0.21 ± 0.62
SS (1%, w/v) + CTN (0.5%, w/v)			
4 hours	5.57 ± 0.11	7.02 ± 0.87	2.01 ± 0.19
12 hours	11.03 ± 1.95	17.59 ± 2.58	4.82 ± 0.18
24 hours	6.93 ± 0.83	3.98 ± 0.82	1.99 ± 0.49
48 hours	3.19 ± 0.82	2.11 ± 1.04	0.99 ± 0.11

All the experiments were carried out in triplicate.

Biochemical estimation

The quantity of CHOL, TGS, or SGE extracted from excised epidermis increased with increase in concentration of SS extract up to 1% (w/v). However, higher concentrations of SS did not significantly ($P < 0.05$) effect the quantity of any skin microconstituent extracted. Treatment with SS extract (1%, w/v) for 48 hours extracted maximum amount of CHOL (18.57%) followed by TGS (11.4%) and least amount of SGE (8.44%).

However, treatment with combination of CTN and SS extract produced maximum perturbation as is evident from highest values of CHOL (23.66%), TGS (17.05%), and sphingosine (12.09%) extracted from excised epidermis. It is interesting to note that all these treatments were significantly less effective ($P > 0.05$) when applied to viable skin. Further, the maximum effect in viable skin was observed when epidermis was excised after 12 hours of application of any formulation (Table 4). Therefore, SS extract, CTN, or combination of SS extract and CTN can be suggested to influence the permeation of CDL through modulation of epidermal barrier status by affecting the skin microconstituents.

Differential scanning calorimetric analysis

Figure 1 depicts the DSC thermogram of rat epidermis after treatment with SS extract. The normal (untreated) epidermis (Figure 1a) exhibited three endothermic transitions each at 70°C (T_2), 80°C (T_3), and 97°C (T_4). Treatment with PG:ETOH (7:3) mixture obliterated the T_4 endotherm (Figure 1b). The T_4 endotherm was also absent in thermograms of excised epidermis treated with aqueous solutions of SS extract (0.5–1%, w/v) (Figure 1d–f) except after treatment with 0.1% SS extract (Figure 1c). Decrease in enthalpy accompanied with slight shifting of T_m to higher temperature T_2 transition was observed after treatment of excised epidermis with any dose of SS extract. Further, the T_m was found to shift toward slightly higher temperature. The broad nature of endotherm and shifting of T_m toward higher temperature might be due to merger of T_2 and T_3 transitions. These effects reveal the effect of the treatments on epidermal lipids. Hence, treatment with SS extract (0.5–1%, w/v) could be suggested to influence both epidermal lipids and proteins.

Transepidermal water loss studies

Figure 2 compares the effect of 1% (w/v) sodium lauryl sulfate (SLS), SS extract (1%, w/v), or SS extract (1%, w/v)–CTN (0.5%, w/v) mixture on water permeability status

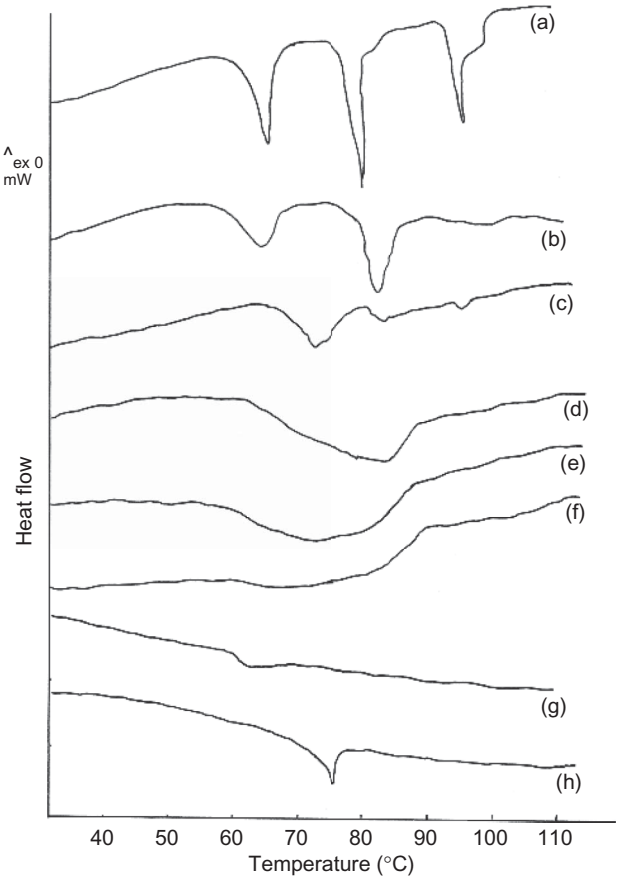

Figure 1. DSC thermograms of normal rat epidermis (a), and after treatment with PG:ETOH, (7:3) (b), SS 0.1% (w/v) (c), SS 0.5% (w/v) (d), SS 0.75% (w/v) (e), SS 1% (w/v) (f), SS–CTN mixture (g), CTN 0.5% (w/v) (h).

of viable rat epidermis. All treatments significantly ($P < 0.05$) increased the TEWL as compared to the control. The maximum TEWL (30.2 g/m^2/h) was observed after application of patch containing SLS (1%, w/v) to viable rat epidermis for 12 hours, and the effect did not normalize even after 48 hours of removal of this patch. The application of patch containing SS extract or combination of SS extract and CTN too increased the TEWL to, respectively, 9.3 and 14.3 g/m^2/h. However, the TEWL started decreasing after 24 hours and completely normalized after 48 hours of removal of these patches. A high TEWL indicates defects in the barrier function of the skin. As the skin barrier function is believed to be primarily located in the intercellular domains, the lipid phase acts as a barrier against water loss. Hence, it seems logical to correlate the increase in TEWL with the permeation of CDL across viable rat skin after treatments with SS extract. Figure 3 depicts a direct bearing of enhanced TEWL of rat epidermis after treatment with SS extract or SS extract–CTN mixture on the permeation of CDL. Abrams et al.[29] studied the effect of solvents (chloroform : methanol mixture, hexane : methanol mixture, etc.) on the lipid extraction and TEWL in human skin in vitro. Most of the solvents caused lipid depletion and an increase in the TEWL. However, no correlation was observed between the amount of lipid extracted and the increase in TEWL. On the contrary, the results of this investigation revealed lipid extraction to be directly reflected as enhanced TEWL at all the time periods. These findings strongly suggested the enhanced permeation of CDL to be ascribable to depletion of epidermal lipids and increased TEWL after treatment with SS extract or SS extract–CTN mixture.

Scanning electron microscopy and transmission electron microscopy studies

SEM photomicrographs revealed untreated rat epidermis to consist of closely united assembly of squames (Figure 4a). Treatment with SS extract (1%, w/v) produced slight loosening of surface layers and small pores in SC (Figure 4b). The treatment with CTN created pores of larger size (Figure 4c). Maximum perturbation due to loosening and creation of pores in upper layers of SC was observed after treatment with combination of SS extract and CTN (Figure 4d). Figure 4e–g depicts the photomicrographs of epidermis excised from skin after 12 hours of treatment with these formulations. Among these samples, although, maximum effect was produced by treatment with SS–CTN mixture; no pore formation in upper layers of SC was evident.

The photomicrographs obtained after TEM revealed greater disordering of skin lipids in excised epidermis after CTN treatment (Figure 5c) as compared to control (Figure 5a) or SS extract treatment (Figure 5b). The treatment with SS–CTN mixture appeared to produce greatest perturbation of excised epidermis as evident from increase in intercellular space, disordered lipid structure, and corneocyte detachment (Figure 5d). Similar treatments to viable skin for 12 hours produced corneocyte detachment and undulation (Figure 5e–g), and the maximum effect was evident after treatment with SS–CTN mixture (Figure 5g). The literature reveals importance of pore formation in epidermis during current assisted transdermal permeation of solutes[30]. However, the density of pores has not been definitely elucidated through complementary morphological studies. The outermost layer of the skin, the SC, is believed to constitute the major barrier for drug permeation and is regarded as a heterogeneous two-compartment system composed of keratin-filled corneocytes, embedded in an intercellular lipid matrix. This lipid

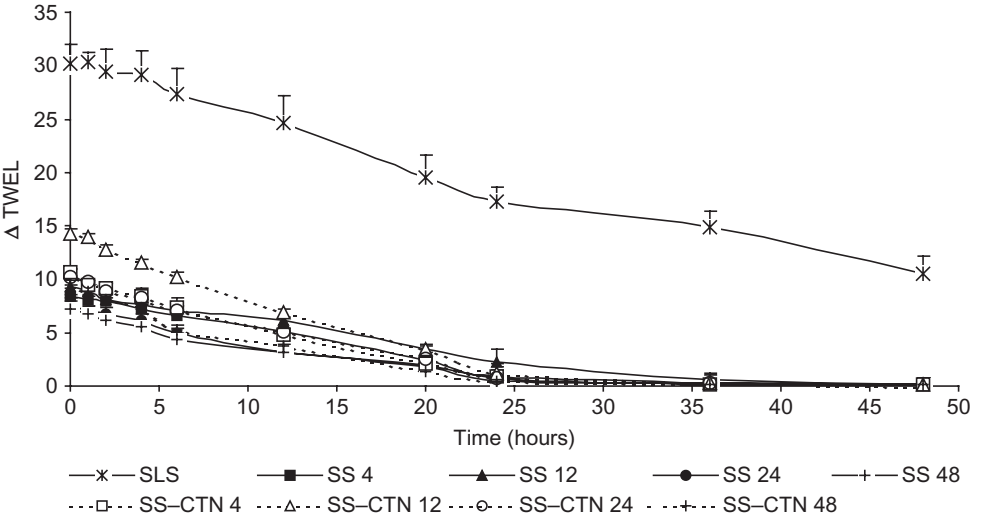

Figure 2. Comparison of effect of treatment of 1% sodium lauryl sulfate (SLS) with SS or SS–CTN mixture on TEWL of epidermis in viable rat skin after different time intervals.

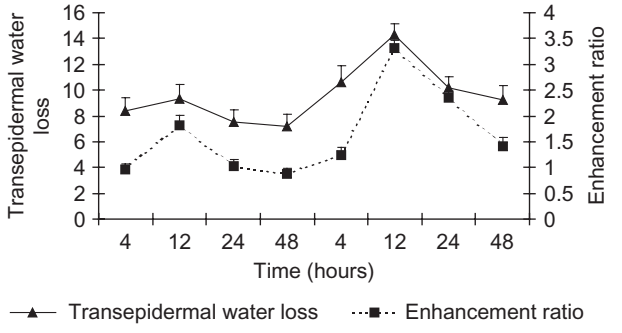

Figure 3. Comparison of TEWL of viable rat epidermis and ER of CDL across rat epidermis excised after treatment with SS extract or SS–CTN mixture for different time periods (TEWL, transepidermal water loss; ER, enhancement ratio, i.e., flux with enhancer/flux without enhancer).

matrix is organized in lamellar bilayers[31]. These bilayers are formed by rearrangement and fusion of lamellar disks that are extruded into the intercellular regions from the uppermost cells of the stratum granulosum[32]. The permeability barrier in the SC is provided by these lipid bilayers and corneocytes[11,33].

Different theoretical and experimental results have suggested that the drug penetration occurs through cavitation-induced keratinocytes or intercellular lipid bilayer disordering. SEM studies revealed loosening and creation of small pores in SC surface layers by SS (Figure 4b), creation of larger pores by CTN (Figure 4c), and greatest loosening and pore formation by SS–CTN (Figure 4d) mixture treatment in excised epidermis. The effect was of apparently less intensity when these formulations were applied to viable skin (Figure 4e–g). The TEM studies also revealed disordering of lipid areas albeit to a smaller extent after treatment of excised epidermis with SS extract (Figure 5c) and increase in intercellular space along with corneocyte detachment after treatment with SS extract–CTN mixture (Figure 5d). These effects were apparently less severe when the treatments were given to viable skin (Figure 5e–g). The overall less intensity of the effects observed in TEM as compared to those observed in SEM suggest the influence of the treatments in deeper skin layers. Nevertheless, the microscopic observations supported the findings of SC microconstituent estimations after similar treatments.

Evaluation of transdermal patches in DOCA-induced hypertensive rats

The results in Figure 6 indicate that the administration of DOCA produced significant hypertension in rats. The oral administration of CDL significantly ($P < 0.05$) controlled the hypertension initially, with the maximum effect observed at 2 hours. However, after 2 hours, the BP started rising gradually until it was the same as the initial value at 24 hours. In contrast, the administration of CDL through transdermal patches resulted in a gradual decrease of BP, with the maximum effect from the patches observed at 4 hours ($P < 0.05$). Despite the fact that the patches produced a peak effect at 4 hours, they decreased the BP significantly ($P < 0.05$) at the first hour and the effect continued for 22 hours. The results of pharmacodynamic study clearly indicate that the transdermal patches released the drug gradually over a period of time, which resulted in prolonged control of hypertension for 22 hours. Orally administered CDL acted quickly and drastically, but then its effect dropped off quickly. On the other hand, the transdermal patches did not decrease the BP greatly in the initial phase in

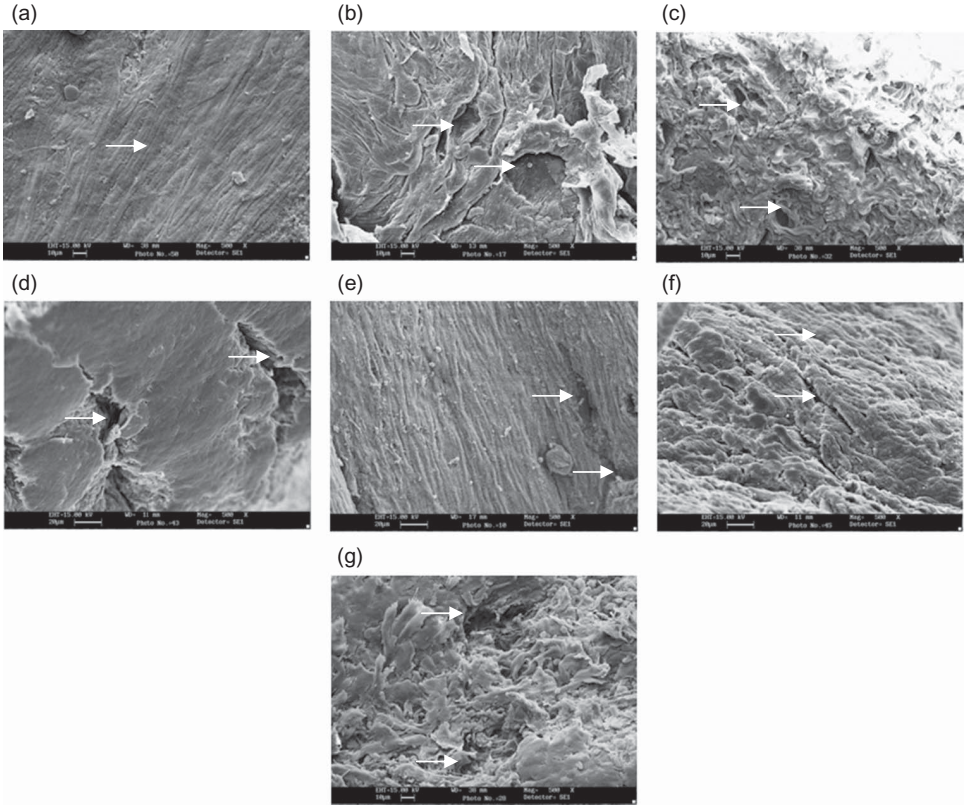

Figure 4. Scanning electron micrograph of excised rat skin: without any treatment (a), after treatment with CTN (b), after treatment with SS (c), after treatment with SS–CTN mixture (d); and viable skin excised after 12 hours of treatment with SS (e), CTN (f), SS–CTN mixture (g) (white solid arrow indicates surface effect on stratum corneum, magnification is 500×, scale bar ⎓ is 10 μm and ⎯ is 20 μm).

comparison to the oral form, as indicated by the significant ($P < 0.05$) difference between the oral- and the patch-treated groups at 2 hours. However, the effect of orally delivered CDL started declining after 4 hours because of its high hepatic metabolism and short half-life. Since, the release of CDL from transdermal patches was sustained through 22 hours, they were able to control the hypertension throughout this period. These results strongly proved the contention that the prepared transdermal patches were capable of overcoming the shortcomings of oral administration of CDL including high first pass metabolism, low bioavailability, and short half-life.

Pharmacokinetic parameters of carvedilol in animals

The maximum plasma concentration (C_{max}) of CDL after oral administration was 121.042 ± 0.98 ng/mL, and T_{max} was 2 hours. The application of transdermal patch containing CDL dispersed in PG:ETOH (7:3) produced C_{max} and T_{max} of 230.33 ± 6.12 ng/mL and 12 hours, respectively. However, application of the transdermal patch containing SS extract–CTN mixture produced C_{max} and T_{max} of 271.46 ± 2.78 ng/mL and 12 hours, respectively (Figure 7). It has been reported that oral clinical doses of CDL produce C_{max} of 23–79 ng/mL[34]. However, Hokama et al.[35] reported C_{max} of 297.7 and 491.7 ng/mL after, respectively, 20 and 40 mg/kg oral administration of CDL to rats. The present investigation revealed attainment of C_{max} of 123.04 ng/mL after 10 mg/kg oral dose of CDL. The observed C_{max} of 123.04 ng/mL is approximately half of that reported by Hokama et al.[35] This seems to be due to half the oral dose of CDL (10 mg) used in this investigation as against 20 mg used by Hokama et al.[35] Although, the C_{max} attained after application of 40 mg dose of CDL in transdermal patches containing SS extract (1%, w/v)–CTN (0.5%, w/v) mixture as permeation enhancer was 271.46 ± 2.78 ng/mL, it was maintained above the C_{eff} concentration for 36 hours. This was manifested in control of hypertension through 30 hours in DOCA-induced hypertensive rats in comparison to 8 hours after oral administration of CDL as observed by Hokama et al.[35] Hence, the results of the present investigation reveal distinct advantage of transdermal delivery of CDL using SS extract–CTN mixture as permeation enhancer as compared to oral administration in terms of duration of activity.

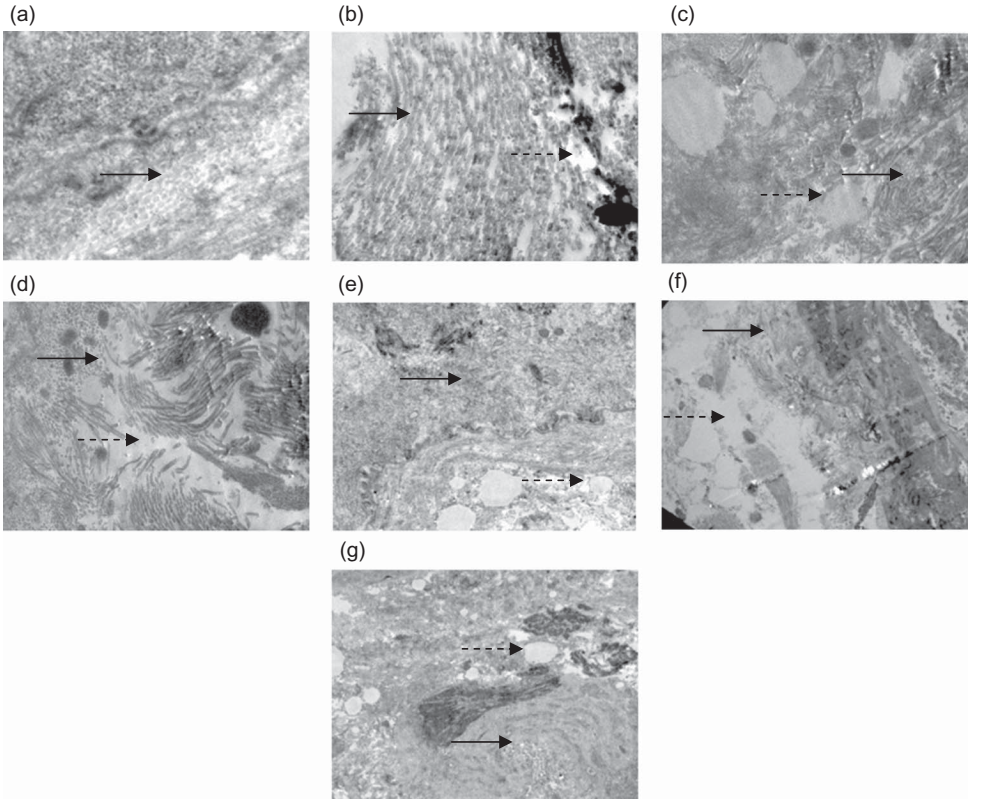

Figure 5. Transmission electron micrograph of excised rat skin: without any treatment (a), after treatment with CTN (b), after treatment with SS (c), after treatment with SS–CTN mixture (d), and viable skin excised after 12 hours of treatment with SS (e), CTN (f), SS–CTN mixture (g) (black solid arrows indicate arrangement of corneocytes and broken arrows indicate lipid perturbation of intercellular lipids, magnification is 20,000×, scale bar ⟵ is 100 nm).

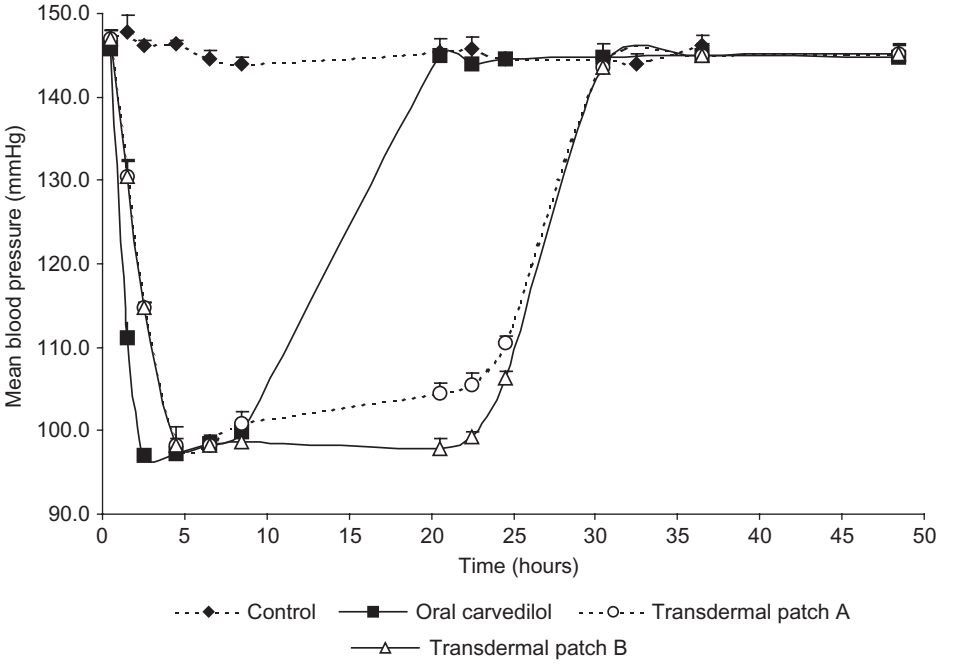

Figure 6. The influence of administration of CDL by oral or transdermal route on blood pressure in DOCA-induced hypertensive rats [transdermal patch A, contains PG:ETOH mixture (7:3); transdermal patch B, contains SS extract–CTN mixture].

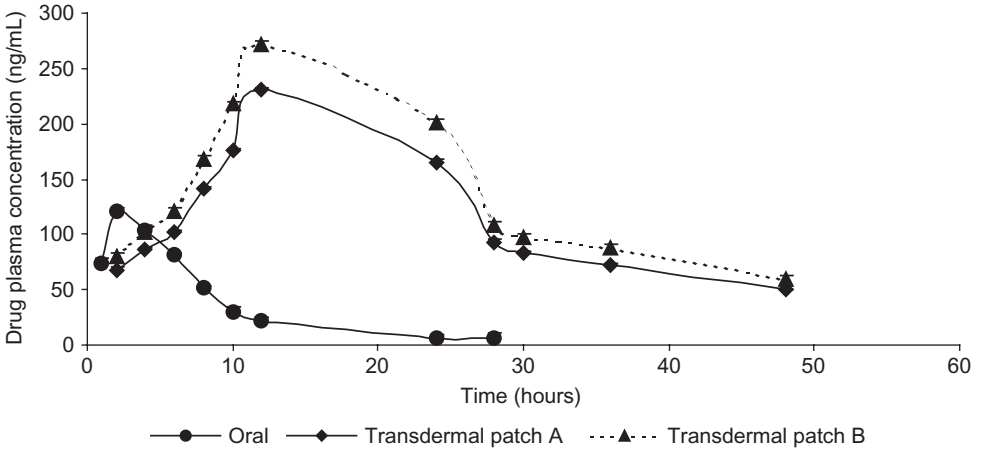

Figure 7. Pharmacokinetic profile of CDL following its administration by oral or transdermal route [transdermal patch A, contains PG:ETOH mixture (7:3); transdermal patch B, contains SS extract–CTN mixture].

Conclusion

The permeation enhancing activity of SS extract and CTN was found to be mediated through their effect on microconstituents of epidermis that was manifested in the ultrastructure as judged by SEM and TEM photographs. Hence, biochemical constitution and modification of epidermal ultrastructure seem to be inextricable aspects while understanding the percutaneous permeation activity of saponins such as SS extract. Further, the normalization of contents of epidermal microconstituents within 48 hours of application of SS extract, CTN, or mixture of SS extract and CTN to viable skin rules out the possibility of irreversible damage by any of these agents.

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