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TRANSDERMAL DELIVERY OF ACECLOFENAC: EFFECT OF PIPERINE AND ITS MECHANISM OF ACTION

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ABSTRACT

Piperine, an amide alkaloid of black pepper, was investigated for transdermal enhancer activity using human cadaver skin in vitro with aceclofenac as the model drug. Furthermore, FT-IR studies were conducted to understand to possible enhancement mechanism. Piperine, at all three concentrations tested, significantly increased flux of the drug compared to control ($p<0.05$). Similarly permeability coefficient (Kp), cumulative amount release (Q24) and enhancement ratio (ER) shown significant increase over control sample whereas skin content of aceclofenac and lag-time of enhancer treated epidermal membrane shown proportionate reduction over control. FT-IR studies reveal that piperine reduces peak area by 19.17 % and 16.87 % for symmetric and asymmetric stretching peaks. In addition, piperine significantly reduces percentage of secondary structures of keratin at amide I band. These results indicate that piperine enhances transdermal permeation of aceclofenac by biphasic mechanism involving partial extraction of stratum corneum (SC) lipid and interaction with SC keratin.

KEYWORDS

Permeation enhancer; Piperine; FT-IR; Aceclofenac; Stratum corneum

INTRODUCTION

Even after wide spread acceptance of modern drug discovery tools such as combinatorial chemistry, High Throughput Screening (HTS), Computer Aided Drug Design the drugs with poor biopharmaceutical properties is yet not reduced. Many of these molecules are very successful and potential therapeutic agents, but suffer from serious drawback of very low bioavailability because of poor permeation across biological barriers[1,2]. Various approaches to reversibly remove the barrier resistance have been investigated. Among these approaches, co-administration of drug with chemical enhancer is widespread accepted and is explored for several drug molecules[3]. Among natural products, one of extensively studied class is essential oils and terpenes. Many mono and sesquiterpenoids have been reported as permeation enhancers. Being natural in origin, terpenes are regarded as relatively safe and clinically acceptable and have been explored as permeation accelerants for many lipophilic as well as hydrophilic drugs[4-9].

Aceclofenac is a NSAID of phenyl acetic acid type. It is frequently prescribed NSAID for minor traumas and soft-tissue inflammation and chronic inflammatory conditions such as rheumatoid arthritis[10, 11].

Piperine, is a major alkaloid of *Piper nigrum* Linn. and *Piper longum* Linn., which are widespread consumed as a spice and medicinal compound since ages[12]. In addition, piperine is also known to improve the oral bioavailability of several drug and nutraceutical molecules[14-16].

MATERIALS AND METHODS

Materials

Aceclofenac was procured as gift sample from Leben Laboratories Pvt Ltd., (India). Piperine used was extracted and purified in our laboratory. All other chemicals used were of analytical grade.

(i) Plant material, isolation of piperine and its standardization

The black pepper fruits were procured from local market and authenticated sample was ground to coarse powder. Piperine was extracted according to the method reported[17]. In brief, 500 gm powder was extracted with ethanol (95%) in Soxhlet extractor. The extract was concentrated using rotatory vacuum evaporator and to the concentrated extract, 70 ml of 10% KOH aqueous solution was added and left overnight. The yellow coloured solids were separated and recrystallized in acetone.

The isolated piperine was standardized by HPTLC (Camag, Switzerland) method[13]. The solutions of reference standard, piperine and isolated compound were prepared in methanol and applied on pre-coated silica gel plates (Merck) using Linomat-V applicator and plate was developed in acetone: hexane (3:2) solvent system. The chromatogram was evaluated densitometrically using winCAT software (scanner 3) and piperine was detected at 342 nm. The purity of isolated piperine was 99.46 % w/v.

(ii) Preparation of epidermis and stratum corneum [18,19]

Full thickness human abdominal skin samples (female age 27 years) were obtained post-mortem from Rural Hospital, Amalner (Maharashtra) India and stored at -20^0C in doubled-sealed evacuated

polyethylene bags. On removal of subcutaneous fat from skin, it was immersed in water at 60^0C for 2 min followed by removal of epidermis.

The stratum corneum (SC) sheet was obtained by floating freshly prepared epidermal membranes (SC side up) on an aqueous solution of trypsin (0.001% w/v) and sodium bicarbonate (0.5% w/v), at room temperature for 3 hr. The SC was removed, thoroughly washed and dried in a vacuum desiccators. The Study was duly approved by IAEC.

(iii) In vitro permeation study [20,21]

The diffusion cells, similar to vertical Franz diffusion cells, with 10 ml and 4 ml capacity of receptor and donor compartments respectively with 2.5 cm^2 diameter (2.2 cm^2 effective diffusion area) were used for permeation studies. The epidermal skin layer was mounted carefully on the lower half of the cell with the epidermis facing upwards. The receptor compartments were filled with 0.1M phosphate buffer (pH 6.8). The prepared diffusion cells, containing the buffer, were equilibrated for 1 hr in a water bath at 37°C, prior to the addition of saturated aceclofenac solution to the donor compartment. The receptor compartment was kept at 37°C and stirred with a magnetic stirrer at 400 rpm. After an hour, 3 ml of freshly prepared saturated solution of the aceclofenac in phosphate buffer (pH 6.8) was added to each donor compartment, which was immediately covered with parafilm, to avoid the loss due to evaporation. To determine the effect of the piperine, the epidermal membranes were immersed in 0.5, 1 and 2% w/v piperine solution prepared in phosphate buffer (pH 6.8) for 24 hr, rinsed and mounted in the diffusion cells. Aliquots of 1 ml were withdrawn periodically and replaced with the same volume of receptor fluid for 24 hr. and analyzed on Shimadzu-1610 UV-spectrophotometer for aceclofenac content according to the method reported. After 24 hr, the skins were removed and analyzed for drug content using a modified method.

(iv) FTIR Study [7]

The circular disc of SC of approximate 1.5 cm diameter was prepared and hydrated in sodium chloride (0.9 % w/v) solution containing antimicrobial agents for 3 days. Before hydration of the SC discs for 3 days, FT-IR (Shimadzu-8400S, Japan) were recorded in the frequency range 400 to 4000 cm^{-1} with 2 cm^{-1} resolution. Each spectrum was an average 10 scans. After 3 days of hydration, these discs were thoroughly blotted over filter paper and IR spectra were recorded. Then SC discs were kept in 5 ml of piperine (2 % w/v) prepared in phosphate buffer solution (pH 6.8) at room temperature for 24 hr. Then after 24 hr the SC discs were thoroughly washed, blotted dry and FT-IR spectra were taken. Each sample served as its own control.

(v) Data analysis [4,22]

The skin flux was determined from Fick's law of diffusion.

$J_{ss} = dQ_r / Adt$

Where Jss is steady-state flux in µg/cm^2 per hr, dQr is the change in quantity of material passing through the membrane into receptor compartment in µg, A is the active diffusion area in cm^2 and dt is the change in time.

The cumulative amount of aceclofenac permeated per unit skin surface area was plotted against time and the slope of linear portion of plot was estimated as steady state flux (Jss). The lag time was determined by extrapolating the linear portion of the abscissa.

The permeability coefficient (Kp) was calculated as

$K_p = J_{ss} / C_v$

Where Cv is total donor concentration of aceclofenac.

Enhancement ratio (ER) was calculated by dividing permeability coefficient of aceclofenac through epidermis treated with piperine by permeability coefficient of aceclofenac through the untreated epidermis.

Statistical analysis

Results are expressed as mean ± SD of at least 6 experiments. The permeation study data and FT-IR data were analyzed by analysis of variance (ANOVA) followed by Dunnet test and paired t-test respectively using GraphPad Prism software (version 5.0). The level of significance was selected as ($p < 0.05$).

RESULTS AND DISCUSSION

Permeation studies

The flux, lag-time, enhancement ratio, permeation coefficient and skin content of aceclofenac drug of untreated and piperine-treated epidermis were summarized in (Fig 1, Table 1). It is evident from results that in vitro permeation of aceclofenac through piperine treated epidermis gives significant increase in permeability coefficient (Kp) of drug at all three concentrations compared to control ($p<0.01$). It enhanced Kp by 1.37, 1.75 and 2.21 folds at 0.5, 1, 2 % w/v piperine respectively.

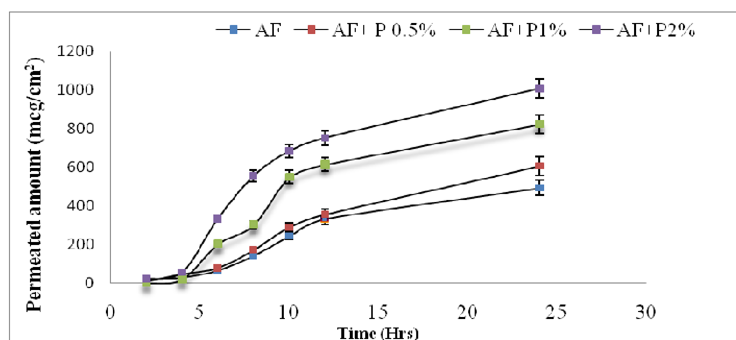

Fig 1

In vitro transport of aceclofenac through human cadaver skin Each data point is the Mean ± SD, (n = 6) (◆) Aceclofenac (AF)- control , (■) Piperine (P)- 0.5% + AF, (▲) Piperine 1% + AF, (●) Piperine 2% + AF.

The corresponding increase in enhancement ratio (ER) was 1.23, 1.66 and 2.01 folds compared to control. However, cumulative amount release at 0.5 % piperine was insignificant ($p>0.05$), where as 1 % and 2 % piperine treatment shown significant rise ($p<0.01$). The flux of aceclofenac was greatest with 2 % piperine treatment, followed by 1 % and 0.5 % and all three concentrations of piperine shown significant increase in flux. The skin content of drug was significantly low ($p<0.01$) compared to control at 1 % and 2 % w/v piperine. Thus, lag-time data and data of skin content suggest that, at 0.5 % w/v piperine treatment no appreciable lipid extraction was observed (refer FT-IR results) and being lipophilic in nature, aceclofenac might be retained in skin, which is evident from skin content of drug which shows insignificant difference between control and piperine treated sample. On increasing piperine concentration at 1 % and 2 % w/v, lipid extraction increased proportionally, which is also reflected in reduction of skin content of drug and also proportionate decrease in lag-time. Thus, increased flux, Kp and reduced lag-time and skin content of drug are better correlated with piperine induced membrane dynamics and permeation characteristic (Refer FT-IR results).

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Table 1

Effect of piperine on transdermal permeation of aceclofenac in vitro human cadaver skin model

Piperine (% w/v)	Flux (µg/cm² per hr)	Lag time (hr)	Q_{24} (µg/cm²)	Kp (10^4)(cm h^{-1})	ER	SC[a] (µg/g)
---	22.56±1.44	0.50±0.8	492.4±57.22	1.07± 0.03	1	199.7±14.4
0.5	27.71± 1.79*	1.36±0.22	605.7±146.5	1.37± 0.14**	1.23	168±12.12
1.0	37.55± 2.02**	0.49±0.10	823.3±70.4**	1.75±0.11**	1.66	146±9.54**
2.0	45.38± 3.36**	0.40±0.12	1008±122.8**	2.21±0.14 **	2.01	128.3±20.2**

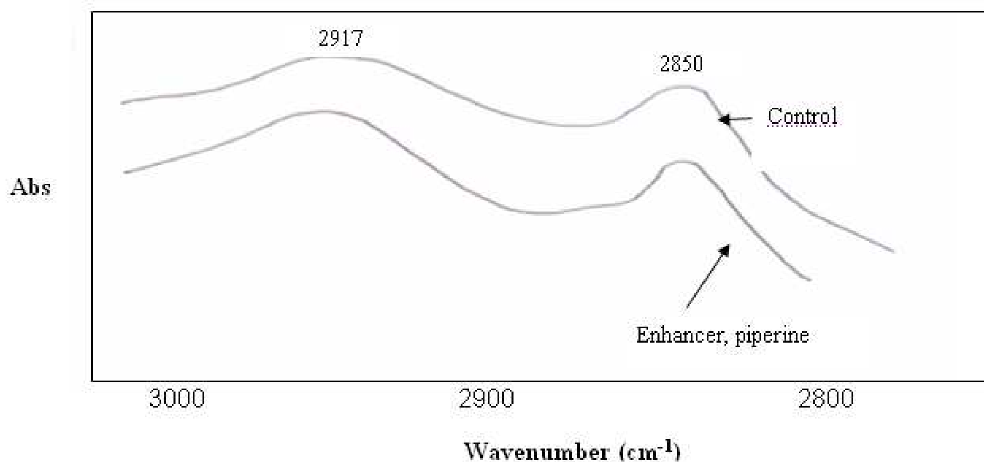
Values are expressed as the mean ±SD, n=6 and
[a] Skin content of drug at 24 hr
**P < 0.05, ** P< 0.01 Dunnet t test vs. control.*

FTIR Study

The biophysical study using FT-IR was undertaken to shade some light on underlying mechanism of enhancement. The molecular vibration of lipids and proteins are related to various peaks of FT-IR spectrum of SC. The band at 2917 cm^{-1} and 2850 cm^{-1} are due to the asymmetric and symmetric CH_2 vibrations of long-chain hydrocarbons of lipids[23,24]. Since the height and area of these two bands are proportional to the amount of the lipids present, any extraction of lipids from SC results in decrease of peak height and area[25]. Further, fluidization of SC lipids also enhances the permeation of drug. The shift of CH_2 stretching peaks to higher wave number (trans to gauche conformation) and increase in their peak widths indicate fluidization of the SC lipids[26,27]. The CH_2 stretching peaks in the spectra of untreated and piperine-treated SC was analyzed for change in peak heights and areas and the shift in of peak frequency after making baseline correction. It was evident from results (Fig 2, Table 2) that treatment of piperine (2 % w/v) reduces both peak height and peak area by 13.18 % and 19.17 % respectively for symmetric C-H stretching (at 2850 cm^{-1}).

Fig 2

FT-IR spectra of SC showing reduction in asymmetric and symmetric CH_2 stretching absorbance after piperine (2%w/v) treatment.



Similar reduction in peak height and area for asymmetric C-H stretching (at 2917 cm^{-1}) by 13.64 % and 16.87 % was also recorded. However, it was also evident form FT-IR spectrum of pure piperine that piperine also gives absorption at both frequency (i.e. at 2850 cm^{-1} and 2920 cm^{-1}). But their intensities are very poor as compared to the spectra of piperine-treated SC and even on subtracting the peak height and area of pure piperine at 2850 cm^{-1} and 2920 cm^{-1} from the spectrum of piperine-treated SC, the peak height and area shown significant difference (paired t-test; p=0.0034 and p=0.0032 for 2850 cm^{-1} and p=0.002 and p=0.001 for 2920 cm^{-1}, peak height and area respectively).

Table 2

The peak height and area of symmetric and asymmetric CH_2 before and after treatment of SC with piperine 24 h and their percentage decrease

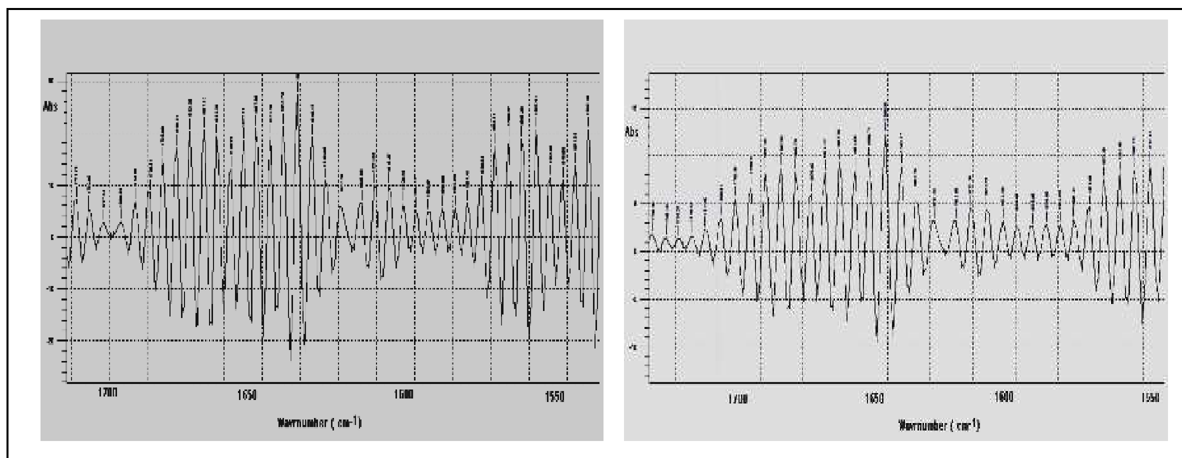
	Symmetric C-H stretching				Asymmetric C-H stretching			
	Peak height	% decrease[a]	Peak area	% decrease[a]	Peak height	% decrease[a]	Peak area	% decrease[a]
Control SC	1.29±0.03	---	167.76±5.05	---	1.32±0.02	---	83.98±2.54	---
Piperine-treated SC	1.12±0.01	13.18**	135.60±4.56	19.17**	1.14±0.02	13.64**	69.81±2.35	16.87**

*Values are expressed as the mean ±SD, n=6 and data analyzed by paired t test vs. control, **P<0.005*

[a]The percentage decrease in peak height or area = (peak height or area of enhancer treated SC – peak height or area of untreated SC) / (peak height or area of untreated SC) × 100.

Thus, although not appreciable, it is clearly indicated that lipid extraction do occur which is relevant to lipophilic nature of piperine. Recently, the effect of piperine on membrane fluidity of rat jejunum using fluorescence spectrophotometer and fluorescent probe pyrene was reported[28]. It was observed that piperine induce disordering of hydrocarbon core and thus increase the membrane fluidity. However, these effects were observed at 5 and 15 min on piperine treatment and decreased and attained normal. Since in present study, FT-IR spectra recorded after 24 h after treatment of piperine and no fluidization of SC lipids was recorded.

The bands at 1650 cm^{-1} and 1550 cm^{-1} are due to the amide I and amide II stretching vibration of SC proteins. The frequencies of these two bands, especially amide I band are sensitive and shift to higher or lower frequencies according to the change in protein conformation[29]. In piperine-treated SC, no such shifts were observed but since amide I band consisting of component bands that represents various secondary structures of keratin and determination of percentage of these secondary structures will be useful parameter to know the extent of interaction with keratin. The percentage of secondary structure was determined by deconvolution of amide I band and curve-fitting analysis of deconvoluted spectra (refer Fig.3 & Table 3). It is evident from results that percentage of secondary structure of β-sheets, anti-parallel β-sheets and β-turns were shown significant reduction (paired t-test, p< 0.0001).



A B

Fig 3

Protein deconvolution and curve-fitting spectra of amide I and amide II bands of untreated SC (A) and SC treated with piperine (B).

In addition, peak at 1625.88 cm^{-1} was not observed in piperine-treated SC (β-sheet region).

Similar reduction of percentage of secondary structures of α-helix and random coils were also recorded ($p< 0.001$). Thus, it is obvious that reduction of the percentage of secondary structure compared with the untreated SC was due to interaction of piperine with keratin. These results are consistent with previous report that piperine interact with hydrophobic portions in the protein vicinity[28]. Taken together, results presented here suggest that decrease in diffusion path length by piperine can be attributed to SC lipids extraction and substantial interaction with SC keratin.

Table 3

The percentage secondary structures of keratin before and after treatment of SC with piperine.

% β-sheet (1620-1640 cm^{-1})		% α-Helix (1650-1660 cm^{-1})		% Anti-parallel β-sheet and β-turns (1660-1695 cm^{-1})		% Random coil (1640-1650 cm^{-1})	
Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
37.09±1.73	18.10±1.46**	62.17±3.72	33 ± 3.20*	140.20±5.32	76.46±2.03**	36.21±1.75	20.14±2.57*

Values are expressed as the mean ±SD, n=6.
**p<0.001, ** p<0.0001(paired t-test vs. control)*

CONCLUSION

The study presented here confirms that piperine induces alteration in membrane dynamics and permeation characteristic of SC by lipid extraction and interaction with keratin and thereby increased permeation of aceclofenac across human epidermal membrane. Thus, easy availability, inexpensive, relatively safe, effective at lower concentration and biphasic mode of permeation enhancement of piperine make it attractive natural molecule for further investigation for various polar and non-polar drugs. Alternatively, it can also be exploited as template or scaffold for development of various analogues and semi-synthetic derivatives with improved efficacy and safety as transdermal permeation enhancer. However, additional biophysical techniques are necessary to elucidate molecular mechanisms of enhancement.

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