Research Article

Effect of Terpenes on the Skin Permeation of Ketoprofen through Shed Snake Skin

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Abstract

The objective of this work was to investigate the effect of hydrocarbon (α-pinene and limonene) and oxygen containing monoterpenes (carvone and terpineole) at 5%w/v in hydroalcoholic mixtures (50% ethanol) on the permeation of ketoprofen across shed snake skin of *Python molurus bivittatus*. The amount of KP retained in the skin after 8 h of diffusion was determined. It was found that the percutaneous absorption of ketoprofen was enhanced in the presence of the enhancers. The rank order of enhancement ratio for skin permeation was found to be α-pinene > limonene > carvone > terpinoele. The enhancers also affected the retention of KP in the skin. The rank order of enhancement ratio for skin retention was the same order with skin permeation experiment. The results indicated that that lipophilicity is an important structural feature for monoterpenes as skin permeation enhancer for a liphophilic drug, ketoprofen.

***Key Words*:** Terpenes; Transdermal delivery; Ketoprofen; Shed snake skin

Introduction

The transdermal route has many advantages for the administration of drugs for local and systemic therapy. However, the outermost layer of skin, the stratum corneum forms a strong barrier to most exogenous substances including drugs. The barrier function of the stratum corneum is attributed to its multilayered wall-like structure, in which terminally differentiated keratin-rich epidermal cells are embedded in an intercellular lipid-rich matrix (Knutson et al., 1985). One approach to deliver an effective dose of drug through skin is to reversibly reduce the barrier function of skin with the aid of penetration enhancers or accelerants (Barry, 1987). Numerous compounds have been evaluated for penetration enhancing activity, including sulfoxides, laurocapram, pyrrolidones, alcohols and alkanols surfactants, and terpenes (Arellano et al., 1998, William and Barry 2004). Terpenes may over advantages over such enhancers because of their low systematic toxicology, high enhancement activity, and low cutaneous irritancy at low concentrations (1-5%) (Okabe et al., 1990; Obata, 1991). Terpenes consist of repeated isoprene(C_5H_8) units, joined together from head to tail. Apart from carbon and hydrogen, terpenes may also contain oxygen such as in carvone, thujone and menthol. Terpenes can be classified depending upon the presence of the number of

isoprene units to be monoterpenes (C_{10}), sequiterpenes (C_{15}), diterpene(C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}), and the chemical groups (i.e. alcohols, esters, ketones) in the structure of terpenes. A variety of terpenes have been investigated as enhancers for both lipohilic and hydrophilic drugs such as 5-fluorouracil (Yamane et al., 1995), tamoxifen (Zhao and Singh, 1998), zidovudine (Narishetty and Panchagnula, 2004), and haloperidol (Vaddi, 2002). However, more information about terpenes enhancing properties in the skin must be investigated to develop effective formulation for transdermal drug delivery. (Cornwell and Barry 1994; Moghimi et al.,1996; Moghimi et al.,1997; Ghafourian et al., 2004).

Ketoprofen, a potent non-steroidal anti-inflammatory drug (NSAID), has been widely used for the treatment of rheumatoid arthritis and related diseases (Kantor, 1986). However, it has adverse side effects including gastrointestinal irritation when administered orally. Since ketoprofen is usually given to patients over an extended period, efforts to reduce its adverse side effects have been attempted. One promising method is to administer the drug via skin. Ketoprofen is an excellent candidate for transdermal delivery among various NSAIDs (Cordero et al., 1997; Vincent et al., 1999), as it has an appropriate partition coefficient and adequate aqueous solubility compared to other NSAIDs. However, its plasma level to therapeutic level via transdermal delivery is limited. Therefore, penetration enhancers have to be included in the formulation for increase percutaneous absorption of enhancers. Various enhancers have been studied for enhancing the penetration of ketoprofen such as propylene glycol, dimethyl sulfoxide, ethanol, polyethylene glycol 400. However, very few data is available for using terpenes as penetration enhancers for ketoprofen.

In this study, the skin permeation of ketoprofen through shed snake skin of *Python molurus bivittatus* was investigated. The effect of hydrocarbon (α-pinene and limonene) and oxygen containing monoterpenes (terpineole and carvone) at 5%w/v in hydroalcoholic mixtures (50% ethanol) as skin penetration enhancer was evaluated. The skin retention of ketoprofen by these terpenes was also assessed.

Materials and Methods

Materials

Ketoprofen (KP) was obtained from Chemio Pharm Company (Rome, Italy). Limonene, α-pinene, terpineole were purchased from Sigma-Chemical Company (St. Louis, USA). Carvone was purchased from Merck Chemicals Company (Darmstadt, Germany). The chemical structure of terpene enhancers is shown in Figure 1.

Solubility studies

The saturated solubility of KP in water, 50% ethanol with or without terpene (5% w/v) enhancer was performed. Saturated solutions were prepared by adding excess drug to the solvents and stirring for 48 h at 37 ˚C. The solutions were then centrifuged, and then the drug concentration was determined by high performance liquid chromatography (HPLC).

In vitro skin permeation

The method of measuring percutaneous absorption followed Test Guideline 428 of the Organization for Economic Cooperation and Development (OECD, 2000). Shed snake skin of *Python molurus bivittatus* was used as a model membrane for skin permeation study because of its similarity to human in terms of lipid content and skin permeability (Hirvonen et al., 1991, Ngawhirunpat et al., 2008). Shed snake skin was donated by the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The shed snake skin was obtained from 3-4 different snakes. Each shed snake skin can be divided into 10-12 pieces. The thickness of shed snake skins was about 0.02-0.03 mm. The skin was mounted between two half cells of a side-by-side diffusion cell with a water jacket connected to a water bath at 37 ˚C, each having 4.0 ml volume and 0.78 cm^2 effective diffusion area. The receiver and donor compartments were filled with

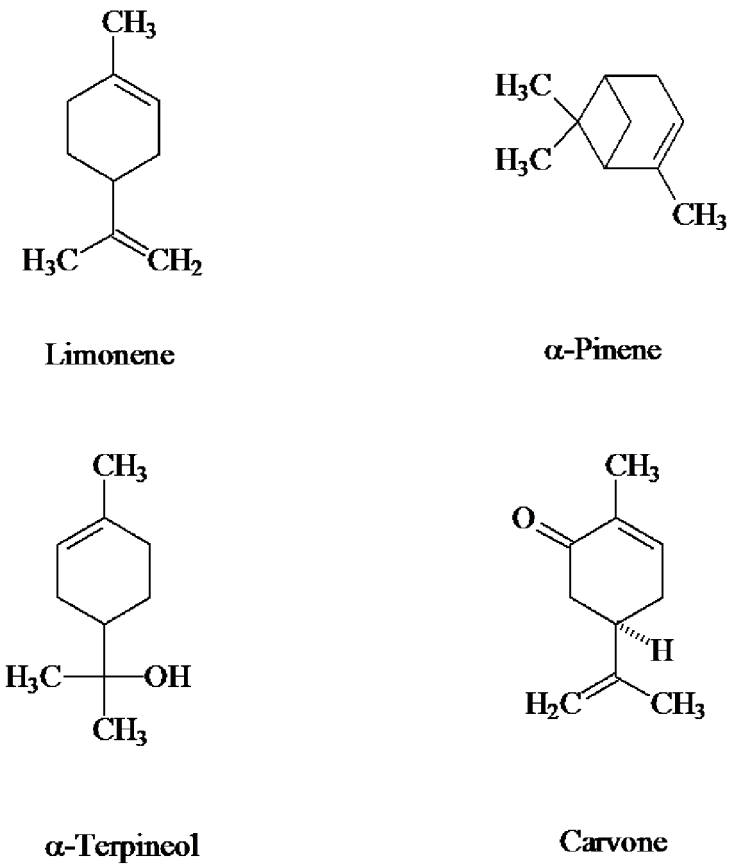

Figure 1 The structural formulae of terpenes used in this study.

distilled water and stirred with a Teflon magnetic stirrer at 600 rpm. After 1 h of equilibration, the receiver compartment was filled with freshly distilled water. The donor compartment was replaced with a saturated solution of drug in 50% ethanol in water, or 5% terpenes in 50% ethanol in water. Skin permeation was run at 37 ˚C for 8 h. A part (1.0 ml) of receiver solution was withdrawn and replaced with the same volume of distilled water to keep the volume constant. The concentration of drugs in all samples was assayed by High Performance Liquid Chromatography (HPLC).

Skin retention study

At the end of the permeation experiment, the exposed skin was washed using 50% ethanol in water, distilled water and blotted dry. The treated skin area was weighed, cut into small pieces, and place in 5 ml of distilled water with occasional stirring for 24 h. The desorbing solution was filtered through membrane filter, and the amount of KP in the filtrate was determined by HPLC.

Analytical methods

The HPLC system consisted of a pump (Perkin Elmer, Massachusetts ,USA), a sperisorb ODS 5-C18, 4.6 mm x 250 mm columm (Scitronic Co. Ltd., Bangkok, Thailand), a variable UV detector (Perkin Elmer, Massachusetts ,USA) and an integrator (Perkin Elmer, Massachusetts ,USA). The mobile phase was composed of 1.0% (v/v) phosphoric acid : methanol (75 : 25). The flow rate of mobile phase was 1.0 ml/min, and the detection wavelength was set at 254 nm. A series of standard solution was prepared to give the final concentration ranging from 1-100 μg/mL. The detection limit was 0.05 μg/mL. The percent relative

standard deviation (RSD) of method precision was 2.0 %. The % RSD of method accuracy was 2.4 %. The correlation coefficient plotted between peak area (Y-axis) and drug concentration (X-axis) was 0.9998.

Data analysis

The cumulative amount of drug permeated through a unit area of skin was plotted as a function of time. Steady state permeation rate or Flux (J_S) and lag time (L) were obtained from the slope and the x-intercept of the linear portion respectively. Permeability coefficient (K_p) were calculated from the enhancement equations (Martin, 1993).

$$K_p = \frac{J_S}{C_d} \qquad (1)$$

where C_d is the saturated solubility of the drug in donor compartment.

The enhancement ratios (ER) were calculated from equation 2 (William and Barry, 1989)

$$ER = \frac{Kp(with\ enhancer)}{Kp(without\ enhancer)} \qquad (2)$$

Analysis of variance (ANOVA) with Dunnett's test in multiple comparison was used for statistical evaluation of the data. *P*-values of < 0.05 were considered to represent a statistically significant difference.

Results and Discussion

***In vitro* permeation studies**

The *in vitro* skin permeation profile of ketoprofen in 50 % w/v ethanol and mixture of 50 % w/v ethanol with 5 % w/v terpene (limonene, α-pinene, carvone, terpineol) through shed snake skin of *Python molurus bivittatus* are shown in Figure 2. In our study, 50% w/v was used as it has been described that high concentration of alcohol (>60%w/v) can reduce the transdermal flux of permeants due to its capacity for dehydrating the skin (Thomas and Panchagnula, 2003). The terpene enhancer concentration was fixed at 5% w/v, taking into account previous reports showing that the enhancement effect is concentration-dependent (Borras-Blasco et al., 1997; Moghimi et al., 1998; Lopez et al., 2000; Nokhodchi et al., 2007). At high concentration of terpene (>5% w/v), the enhancement effect does not increase proportionally, but may instead reach a plateau level (Borras-Blasco et al., 1996). The cumulative amount of KP permeated increased linearly with time after a short lag time (0.1-0.3 h). The solubility of KP, steady state permeation rate (Flux), lag time (L), permeability coefficient (K_p), enhancement ratio (ER) of KP through the shed snake skin, and amount of KP retained in the skin are summarized in Table 1. The 50 % ethanol significantly increased about 2.4-fold KP flux relative to water (data not shown). In this study, 50 % ethanol was used to solubilize terpenes, therefore, the ER was calculated between the ratio of K_p of KP in terpenes plus 50 % ethanol and K_p in 50 % ethanol as control. Our results confirm the capacity of ethanol to permeate through the skin due to its keratin solubilizing effects and altering the organization of stratum corneum intercellular lipids, therefore, increasing skin permeability (Knutson et al., 1990; Bergstrom et al., 1990).

In the presence of ethanol and absence of terpene enhancer, the transdermal flux of KP was 6.9 $\mu g/cm^{-2}\ h^{-1}$ (Table 1). The presence of terpene enhancers produced a 1.3- to 2.8-fold flux increase over only ethanol as a vehicle. The permeation lag times ranged from 0.1-0.3 h, without statistically significant differences among the individual vehicles. Takahashi et al. (1993) reported that the lag time of neutral, acidic and basic compounds with molecular weight below 200 was not observed when using shed snake skin of *Elaphae obsoleta* as a model membrane. The molecular weight of the KP was over the range 254. However, the lag time of shed snake skin was found to be small in both with and without enhancers. The percutaneous penetration flux and permeability coefficient were increased with the terpene enhancer in the following order: α-pinene > limonene > carvone > terpineol. The enhancement ratio of α-pinene and limonene showed an

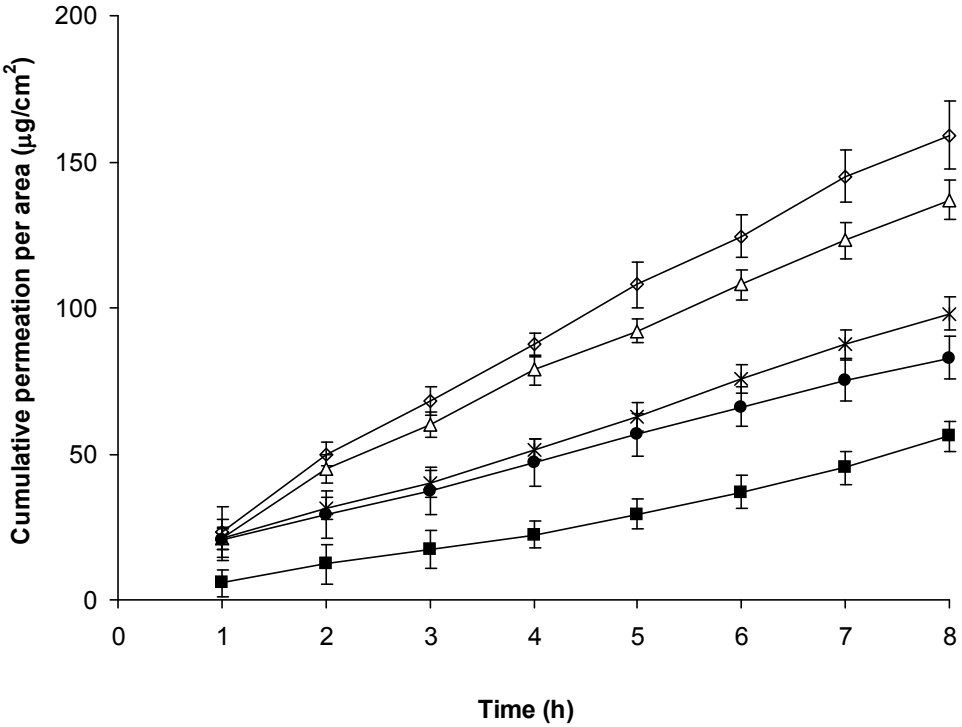

Figure 2 Permeation profiles through shed snake skin of KP from various vehicles.
(■) 50 % w/v ethanol, (●) 50 % w/v ethanol with 5 % w/v terpeniole,
(×) 50 % w/v ethanol with 5 % w/v carvone, (△) 50 % w/v ethanol with 5 % w/v limonene,
(◊) 50 % w/v ethanol with 5 % w/v α-pinene.
Each point represents the mean ± S.D. of three to five experiments.

enhancement ratio of 2.8 and 2.3 compared with control, repectively, whereas carvone and terpineol showed an enhancement ratio of 1.6 and 1.3 compared with control. α-pinene and limonene are hydrocarbon terpene, they contains hydrophobic tail and therefore have no a hydrogen bonding group, whereas carvone and terpeniole are ketone and alcohol terpene, repectively, and they have hydrogen bonding group. Our results were consistent with previous reports (Okabe, 1990; Narishetty, and Panchagnula 2004) that for hydrophilic compounds such as 5-fluorouracil and propanolol hydrochloride, hydrophilic terpenes containing functional moieties with hydrogen bonding ability, are effective in enhancing skin transport, whereas for hydrophobic drugs, such as indomethacin, lipophilic terpenes such as limonene, terpinene were found to be most effective.

The possible mechanism of terpenes in skin disruption may be due to the lipid fluidizing activity of terpenes (William and Barry, 1991). It has been reported that terpenes extract lipids from stratum corneum (Ogiso et al, 1995; Zhao and Singh 2000) resulting in disorder of lipid domains, and it was proved by fourier transform infared (FTIR) studies, where there was a decrease in heights and area of both symmetric and asymmetric CH_2 stretching absorbance peaks of stratum corneum lipids (Vaddi et al, 2002; Zhao and Singh 2000). The differences in permeation enhancement ratio of various terpenes may be attributed to the presence of different terpenes with variable molecular weight and boiling points. The higher enhancement of α-pinene and limonene may be attributed to their low boiling point and molecular

Table 1 Steady state permeation rate (Flux), lag time (L), permeability coefficient (Kp) and enhancement ratio (ER) of ketoprofen through shed snake skin.[a]

Vehicles	Solubility[b] (mg/ml)	Flux ± SD ($\mu g\ cm^{-2}\ h^{-1}$)	L (h)	Kp (cm^2/ h)	ER	KP retained in the skin (μg), (% compared with drug in donor solution)
50% w/v Ethanol	1.20 ± 0.02	6.92 ± 0.19	0.28	0.57 ± 0.03	1.00	16.35 ± 2.23 (0.45 %)
50% w/v Ethanol+ 5% w/v Terpineol	1.29 ± 0.03	9.16 ± 0.41*	0.11*	0.71 ± 0.06*	1.32 ± 0.24*	17.22 ± 3.31 (0.44 %)
50% w/v Ethanol+ 5% w/v Carvone	1.31 ± 0.05	11.16 ± 0.50*	0.12*	0.85 ± 0.05*	1.61 ± 0.30*	19.23 ± 3.51 (0.49 %)
50% w/v Ethanol+ 5% w/v Limonene	1.29 ± 0.04	16.21 ± 0.54*	0.07*	1.22 ± 0.08*	2.34 ± 0.18*	25.44 ± 3.22* (0.66 %)
50% w/v Ethanol+ 5% w/v α,-Pinene	1.28 ± 0.04	19.22 ± 0.64*	0.06*	1.49 ± 0.09*	2.77 ± 0.19*	29.78 ± 3.25* (0.78 %)

a) Each point represents the mean ± S.D. of three to five experiments.

b) at 37 ˚C .Each point represents the mean ± S.D. of three to five experiments.

* $P < 0.05$ compared with 50% w/v ethanol.

weight. The low boilng points of terpenes indicate the weak cohesiveness or self-association of the molecules (Martin et al, 1993) and therefore they may more easily associate or interact with lipid components of stratum corneum and alter the barrier property.

Skin retention study

When 5% w/v terpenes were incorporated with 50% w/v ethanol, the skin retention of KP was higher than the values obtained for only 50% w/v ethanol (Table 1), indicating terpenes affected the retention of KP in the skin. The amount of KP retained in the skin was in the following order: α-pinene > limonene> carvone ≈ terpineol. These data confirmed that KP retention was related to flux values across the skin. Terpenes enhancers affected the retention of LHRH in the skin (Songkro et al, 2009). Moreover, Koyama (1994) reported that skin concentration after epidermal application of several drugs could be related to their flux values across epidermis.

Conclusion

In this study, the permeation of KP through shed snake skin is significantly enhanced by 5 % w/v of terpenes. The addition of terpenes enhancer in the formulation was also found to increase KP retention. Among the terpenes tested, α-pinene was the most effective penetration enhancers. Our results indicated that that lipophilicity is an important structural feature for monoterpenes as skin permeation enhancer for a ketoprofen. These results indicated that terpenes at low concentration (5% w/v), especially α-pinene can be used in pharmaceutical products as ingredient and penetration enhancer.

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