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Combination strategies to enhance transdermal permeation of zidovudine (AZT)

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The objective of this study was to evaluate the effect of simultaneous application of two penetration enhancers of different chemical classes or a chemical penetration enhancer and current application on permeation of zidovudine (AZT) across rat skin. *Ex vivo* permeation of AZT using combinations of cineole or menthol in vehicle with either oleic acid/linolenic acid or 0.5 mA/cm^2 anodal current application for 6 h was studied. Penetration enhancers were significantly different in enhancing the permeability of AZT across rat skin and are in the decreasing order of activity: linolenic acid > menthol > oleic acid > cineole > vehicle. The combination of cineole and oleic acid synergistically enhanced transdermal flux of AZT in addition to reducing lag time. However, this was not observed for combinations of menthol with oleic or linolenic acid. On the other hand, the simultaneous application of current with menthol and cineole significantly increased cumulative amounts of AZT permeating during the course of current application and reduced the lag time but failed to further increase steady state flux of AZT. These results suggest that a combination of two penetration enhancers of different classes or the simultaneous use of iontophoresis and a penetration enhancer may be advantageous to achieve permeation enhancement with low risk of skin damage.

1. Introduction

In recent years, transdermal drug delivery systems have emerged as one of the most successful non-oral controlled release systems with a worldwide market share of more than 2 billion pounds. Major advantages of transdermal delivery include predetermined release rate, avoidance of hepatic first pass metabolism, less frequent dosage regimen and side effects. However, transdermal delivery has always been challenged by the formidable barrier property of *Stratum corneum* (SC). As a result, only those drug molecules having low dose (< 10 mg/day), molecular weight (< 500 Da), melting (m. p. < 200 °C), and high lipophilicity (log P > 3) are successfully delivered transdermally. Nevertheless, a large number of drugs remain for which transdermal delivery is desirable but not feasible due to the impermeablity of skin towards them. Several approaches including vehicles, vesicles, supersaturated systems, chemical enhancers, iontophoresis, ultra-sound, microneedles have been suggested to overcome the barrier nature of SC and to deliver many drug molecules across skin [1–5]. Yet, the highest strength of physico-chemical forces that need to be applied to deliver required drug doses is likely to be limited by their adverse physiological effects [6]. Recent trends in transdermal delivery include the simultaneous application of more than one physico-chemical force using two different mechanisms, which may decrease the required strength of these forces to achieve a given transdermal permeation enhancement [7, 8].

Terpenes and fatty acids, which are chemically different, have been extensively studied as penetration enhancers for the improving permeation of several drugs with varying lipophilicity [9–13]. Terpenes act at polar head-group regions of the lipid bilayer [14–16] while fatty acids are known to act at the hydrophobic region either by formation of separate domains [17] or fluidizing the lipid alkyl chains [11, 18]. On the other hand, iontophoresis enlarges existing pore pathways in hair follicles [19] or creates new aqueous pathways by hydrating the intercellular lipid region [20]. As the site and mechanism of action of terpenes, fatty acids, and electric current are different from each other, their combination may lead to synergistic enhancement in permeation of drugs.

Zidovudine (AZT) is widely used in the treatment of AIDS and HIV infections. Its therapeutic use has led to a decrease in mortality rate and frequency of opportunistic infections in AIDS patients [21]. Due to its short half-life and dose related side effects, conventional peroral delivery leads to severe hematological side effects and needs frequent administration leading to poor patient compliance. Transdermal delivery of this molecule can improve its side effect profile and patient compliance. However, due to the hydrophilic nature of AZT, its permeation across the skin is very poor and below the rate sufficient

to achieve therapeutic effects. In previous studies, we have reported an increased permeation of AZT across the skin with various vehicles [5, 22] and terpenes at 5% w/v in vehicle [23]. Moreover, menthol and cineole were also found to be very effective penetration enhancers for improving transdermal permeation of hydrophilic drugs [24] such as imipramine hydrochloride [16], 5-fluorouracil [10] and diclofenac sodium [25]. The objective of the present study was to investigate the effect of simultaneous use of terpenes and fatty acids at lower concentrations in optimized vehicle (66.6% ethanol in water) on transdermal permeation of AZT. In addition, the effect of electric current on the enhancement activity of terpenes was also determined.

2. Investigations, results and discussion

Permeation profiles of AZT across rat skin in presence of terpenes, fatty acids and their combinations are shown in Fig. 1, while permeation parameters such as steady state flux, lag time as well as enhancement ratio are listed in Table 1. All penetration enhancers studied and their combinations except cineole significantly enhanced ($p < 0.05$) the trandermal flux of AZT in comparison to vehicle. These results are in agreement with the literature reports where oxygen containing terpenes were shown to increase skin permeation of several polar drugs such as diclofenac sodium [25], 5-flurouracil [10], propranolol hydrochloride [26] and imipramine hydrochloride [16]. When penetration enhancers were studied individually at 2.5% w/v in vehicle, the order of enhancement was as follows: linolenic acid $\approx$ menthol $>$ oleic acid $>$ cineole $>$ vehicle. This permeation enhancement activity of terpenes may be attributed to several mechanisms i.e. formation of permeable defects due to lipid extraction [27, 28], fluidization of SC lipids [10], breaking of interlamellar hydrogen bonding network [16] and thereby formation of new polar pathways [14, 15, 23]. In contrast, mechanism of fatty acids in general and oleic acid in particular was reported to be due to fluidization of lipid alkyl chain packing [18] and formation of phase separated liquid domains [29].

As seen from Table 1, significant differences in the enhancement abilities of various individual penetration enhancers were observed i.e. ER1 of cineole was 3.2 in comparison to 52.6 of menthol and ER1 of oleic acid was 24.2 against 52.7 of linolenic acid. In previous studies, menthol was found to be an effective penetration enhancer at very low concentrations as compared to cineole and the difference was attributed to the possible differences in thermodynamic activity of the enhancers in the vehicle [23]. On the other hand, the large difference in enhancement abilities of oleic acid and linolenic acid may be due to a variation in the degree of unsaturation. In general, an increase in the number of double bonds among unsaturated fatty acids results in greater skin perturbation [11] due to increased kink in packing geometry of fatty acids thus leading to an enlargement of free volume in SC lipid packing. Accordingly, linolenic acid having two double bonds showed greater enhancement in AZT flux than oleic acid which has only one double bond.

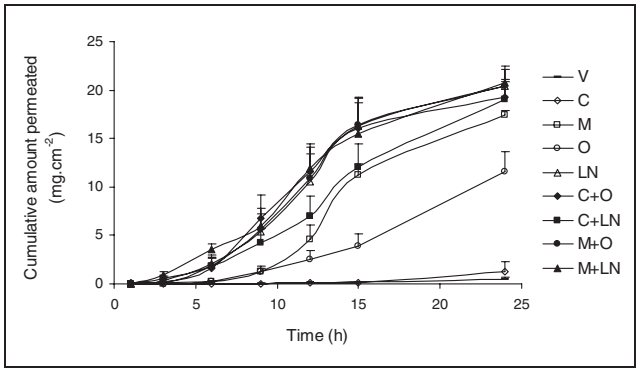

Fig. 1: Permeation profiles of AZT with various penetration enhancers and their combinations at 2.5% w/v in vehicle. Each data point is mean of 4 determinations. Error bars indicate SD. V, vehicle (66.6% ethanol in water); C, cineole; M, menthol; O, oleic acid; LN, linolenic acid

Table 1: Permeation data of AZT arcoss rat skin with various combinations of penetration enhancers[a]

	Flux ($\mu g \cdot cm^{-2} \cdot h^{-1}$)	Lag time (h)	ER 1[b]
Vehicle	32.0 (9.6)	8.9 (0.7)	1[c]
C	102.1 (87.1)	11.8 (0.7)	3.2 (2.7)
M	1681.2 (100.1)	8.6 (0.6)	52.6 (3.1)
O	728.9 (126.1)	8.7 (1.4)	24.2 (5.6)
LN	1686.2 (85.1)	5.8 (2.4)	52.7 (2.7)
C+O	1689.3 (238.7)	5.1 (0.8)	52.8 (7.5)
C+LN	1983.1 (140.3)	8.9 (1.8)	62.0 (4.4)
M+O	1626.3 (162.7)	5.5 (1.1)	50.9 (5.1)
M+LN	1461.1 (275.5)	4.6 (0.7)	45.7 (9.7)

[a]Data is mean of 4 determinations. Standard deviation is indicated in parenthesis. [b]ER 1 = enhancement radio = AZT flux with penetration enhancer/AZT flux without penetration enhancer. [c]ER 1 for vehicle was considered as 1, because the flux of AZT with vehicle was used in the denominator. Vehicle = 66.6% v/v ethanol in water, C = cineole, M = menthol, O = oleic acid, LN = linolenci acid, All penetration enhancers were used at 2.5% v/v in vehicle

Combinations of cineole and oleic acid showed synergistic enhancement in transdermal permeation and significant ($p < 0.05$) reduction in lag time to reach steady state flux. On the contrary, cineole in combination with linolenic acid has shown an additive effect on flux with no effect on lag time values. However, menthol in combination with either oleic acid or linolenic acid did not show any additional enhancement in permeation but the lag time to reach steady state AZT flux was significantly reduced ($p < 0.05$). These differences between cineole and menthol in achieving synergistic enhancement in combination with fatty acids can be attributed to the fact that menthol at 2.5% caused a maximum disorder in SC lipid lamellae thus the effect of fatty acids was not evident in already fluidized lipid lamellae. Nevertheless, it seems that oleic acid facilitates diffusion of menthol into the SC and consequently steady state flux was reached early in their combination. In contrast, cineole at 2.5% minimally alters the barrier properties and therefore in the combination, effects of oleic acid or linolenic acid were clearly evident. In spite of increased perturbations, linolenic acid did not have any additional benefit both in terms of flux enhancement and reduction in lag time when combined with terpenes. Because an increase in lipid perturbation by polyunsaturated fatty acids may not always result in an increased permeation of drugs as drug molecules and co-administered penetration enhancers may be trapped within kinks of polyunsaturated fatty acids nullifying the increased perturbation effect.

Transdermal iontophoresis employs an electric potential as the driving force to enhance skin permeability of charged molecules. Under the influence of this electric potential gradient, 'permselectivity' of skin leads to electroosmosis of charged and neutral molecules through pore pathways

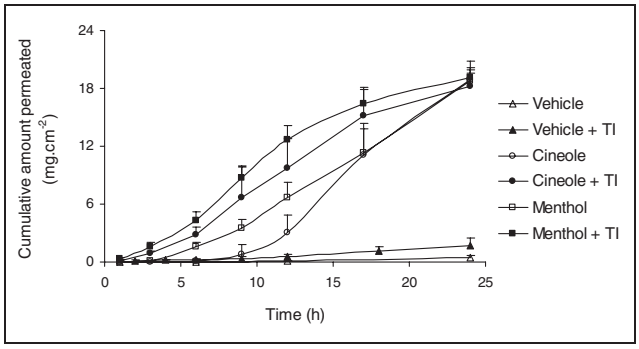

Fig. 2: Permeation profiles of AZT from vehicle in presence of terpenes at 5% w/v with or without current application. Each data point represents mean of 4 determinations. Error bars indicate SD. Vehicle, 66.6% ethanol in water; TI, transdermal iontophoresis (anodal current density of 0.5 mA/cm^2 for 6 h)

[30]. This phenomenon was exploited to deliver neutral and polar molecules such as AZT. Further, as skin is negatively charged at physiological pH, the electroosmotic flow goes predominantly from anode to cathode [31] and hence, anodal current was applied to the donor compartment. At the same time, the magnitude of enhancement increases with current strength and duration of current application, while irreversible skin damage takes place at higher current strengths [32]. From this viewpoint, a current density of 0.5 mA/cm^2 for 6 h duration, which is generally considered as safe and effective for many drugs, was selected in this investigation and further optimization of these two parameters was not done. Fig. 2 illustrates various permeation profiles of AZT in vehicles with or without cineole, menthol and current application. Transdermal iontophoresis increased AZT flux across rat skin threefold in comparison to its passive flux from the vehicle (Table 2) but did not significantly influence enhancement activity of cineole and menthol. However, as shown in Fig. 2, the cumulative amount of AZT permeated was significantly higher, 22 to 26-fold with cineole and 3 to 14-fold with menthol, during the current application period and the differences in the cumulative amounts permeated tapered as the time lapsed after current application. Since the drug molecule does not carry any current, enhancement in its flux during iontophoresis may be explained based on the phenomenon called electroosmosis. Additionally, current application also increases the volume flow of water into skin resulting in increased hydration, which in turn increases the skin permeability. Accordingly, the enhancement in AZT flux during current application period may partly be ascribed to hydration effects. To delineate the effects of penetration enhancer and transdermal iontophoresis from total enhancement achieved, three different enhancement ratios ER1, ER2 and ER3 were calculated which indicate effects of enhancer plus iontophoresis, enhancer in vehicle, and iontophoresis alone. As evident from different enhancement ratios seen in Table 2, iontophoresis showed a significant effect on the permeation of AZT in the absence of terpenes (ER1 with vehicle indicates effect of iontophoresis alone). On the other hand, when applied simultaneously with terpenes, the role of iontophoresis in the combined enhancement was negligible (ER3 with cineole and menthol are close to 1). Nevertheless, lag times were shortened in the presence of iontophoresis indicating that current application increases the transport of penetration enhancers by electroosmosis and consequently early steady state flux values of AZT were achieved.

In conclusion, a cineole-oleic acid combination showed a synergistic enhancement in the transdermal flux of AZT across rat skin. Further, combinations of terpenes and fatty acids reduced the lag time required to reach steady state flux. Moreover, the combination strategy demonstrated reduced skin irritation to rats in comparison to higher concentrations of individual penetration enhancers and the results of this study will be published separately. Similarly, current application in combination with chemical penetration enhancers significantly increased the cumulative amount of AZT permeating during the period of current application and reduced the lag time required to reach the steady state flux. These results suggest that a combination of two different penetration enhancers may be effectively used not only to enhance the permeation rate of drugs and to reduce lag time but also to possibly reduce skin irritation.

3. Experimental

3.1. Materials

AZT was kindly supplied by Cipla Ltd. (India). ^{14}C-AZT, cineole, oleic acid, linolenic acid and sodium azide were purchased from Sigma Chemical Co., USA. Menthol and absolute ethanol were procured from Merck KGaA, Germany. A biodegradable scintillation cocktail was obtained from Amersham Corp., USA. All other chemicals were of reagent grade and were used without further purification.

3.2. Preparation of full thickness skin

The Institutional Animal Ethics Committee of NIPER approved the protocol for the preparation of full thickness skin. After sacrificing the animals (SD rats) with excess ether inhalation, hair on dorsal side was removed with a hair clipper (Aesculap, Germany) taking extreme precaution not to damage skin. The shaved skin was then excised from the animal, subcutaneous tissue was surgically removed and the dermal-side was wiped with isopropyl alcohol to remove any adhering fat [22]. Subsequently, the full thickness skin was washed with phosphate buffer, wrapped in aluminum foil and stored at −20 °C until further use (used within 2 weeks of preparation).

3.3. Ex vivo permeation studies

Ex vivo permeation studies were carried out using unjacketed Franz diffusion cells (Permegear, USA) with a diffusional surface area of 0.785 cm^2 and 5.2 ml of receptor cell volume, placed in heating stirring module [33]. The

Table 2: Permeation parameters of AZT across rat skin with penetration enhancers and iontophoresis[a]

	Flux (μg · cm^{-2} · h^{-1})		Lag time (h)		ER1	ER2	ER3
	Without TI	With TI	Without TI	With TI			
Vehicle	32.0 (9.6)	94.1 (43.9)	8.9 (0.7)	6.1 (1.1)	2.9 (1.4)	–[b]	–[b]
Cineole	1785.7 (324.1)	1931.6 (262.4)	9.0 (1.8)	5.7 (1.7)	60.4 (8.2)	20.5 (2.8)	1.08 (0.1)
Menthol	1599.3 (109.4)	1667.7 (121.7)	7.6 (1.6)	3.0 (0.5)	52.1 (3.8)	17.1 (1.3)	1.04 (0.1)

[a]Data is mean of 4 determinations. Standard deviation is indicated in parenthesis. [b]Not applicable. ER1 = flux with penetration enhancer + TI/flux with vehicle (indicator for combined effect of TI and penetration enhancer). ER2 = flux with penetration enhancer + TI/flux with vehicle + TI (indicator for the effect of penetration enhancer alone). ER3 = flux with penetration enhancer + TI/flux with vehicle + penetration enhancer (indicator for the effect of TI in isolation). A constant anodal TI of 0.5 mA/cm^2 was applied for 6 h using platinum electrodes. Terpenes were present at 5% w/v in vehicle (66.6% ethanol in water). Permeation studies were carried out using Franz type diffusion cells using phosphate buffered saline as receptor medium maintained at 37 °C. AZT was present at 50 mg · ml^{-1} concentration in vehicle.

receptor compartments were filled with phosphate buffered saline pH 7.4 (containing 0.02% w/v of sodium azide) and maintained at 37 ± 0.5 °C. Full thickness skin pieces were mounted over diffusion cells with the dermal-side in contact with the receptor phase, equilibrated for 2 h, and then air bubbles were removed. Subsequently, donor compartments were filled with 400 µl of 50 mg/ml drug in vehicle, with or without penetration enhancers and covered with parafilm to prevent evaporation of vehicle. Samples (200 µl) were withdrawn at specified intervals from the receptor compartment followed by replacement with fresh receptor solution. At the end of the study, receptor samples were mixed with 5 ml of biodegradable liquid scintillation cocktail. Radioactivity in the samples was then measured by a liquid scintillation counter (Wallac 1409, Finland) and drug concentrations were calculated from the ratio of labeled to unlabeled AZT.

In the iontophoretic studies, an anodal constant current density of 0.5 mA/cm^2 for 6 h was applied by placing the anode in the donor compartment and the cathode in the receptor compartment [34]. Current was generated with a custom made 6-channel constant power source (Ultrapure Scientifics, India) through platinum electrodes, while current was adjusted using a multi meter (Hewlett Packard, USA). In the combination of iontophoresis with chemical penetration enhancer strategy, skin was simultaneously treated with penetration enhancer and current for the initial 6 h and current was ceased thereafter.

3.4. Data analysis

The cumulative amount of drug permeated through an unit area of skin was plotted as a function of time. Steady state flux and lag time were obtained from the slope and the x-intercept of the linear portion, respectively.

To compare the permeation enhancement capacities of penetration enhancers, and iontophoresis enhancement ratios were calculated as follows:

$$\mathrm{ER1} = \frac{\text{Flux with penetration enhancer with or with out TI}}{\text{Flux with vehicle}} \quad (1)$$

$$\mathrm{ER2} = \frac{\text{Flux with penetration enhancer} + \text{TI}}{\text{Flux with vehicle} + \text{TI}} \quad (2)$$

$$\mathrm{ER3} = \frac{\text{Flux with penetration enhancer} + \text{TI}}{\text{Flux with vehicle} + \text{penetration enhancer}} \quad (3)$$

Where TI is transdermal iontophoresis (anodal current density of 0.5 mA/cm^2 for 6 h was applied). ER1 indicates the combined effect of vehicle, penetration enhancer and/or transdermal iontophoresis on permeation of AZT, while ER2 separates the effect of vehicle from that of combined enhancement and hence, indicates the permeation enhancement achieved only by terpenes. Similarly, ER3 indicates the role of iontophoresis in total enhancement achieved in AZT flux.

Data of this investigation was statistically analyzed by applying one-way analysis of variance (Tukey multiple comparisons) at a significance level of $p < 0.05$.

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