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Unsaturated cyclic ureas as new non-toxic biodegradable transdermal penetration enhancers. II. Evaluation study

Ooi Wong [1], N. Tsuzuki [2], B. Nghiem [1], J. Kuehnhoff [1], T. Itoh [1], K. Masaki [2], J. Huntington [1], R. Konishi [2], J.H. Rytting [1] and T. Higuchi [1]

[1] *Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045 (U.S.A.) and* [2] *Teikoku Seiyaku Co., Kagawa (Japan)*

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Summary

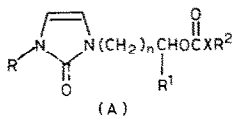
Thirteen new transdermal penetration enhancers were evaluated with respect to the absorption of indomethacin using shed snake skin of *Elaphe obsoleta* as a model of the stratum corneum and using Azone as the standard enhancer for comparison. Indomethacin was incorporated into a petrolatum ointment together with the appropriate enhancer. Three of these compounds, (A1), (A2), and (A3), show penetration enhancement at least equal to or better than Azone. Using hairless mouse skin as the model skin, (A1) shows enhancement 3 times better than Azone. Hairless mouse skin is more permeable than snake skin by indomethacin. The low toxicity of the enhancers was demonstrated by the survival of mice which were given s.c. a total of 14 g/kg of (A2). The biodegradability of the enhancers was illustrated by the hydrolysis of (A3) with porcine esterase in isotonic phosphate buffer pH 7.2 at 32°C with a $t_{1/2} = 7.28$ min. A release study of indomethacin for a series of ointments consisting of a variable concentration of indomethacin indicates that the saturated concentration of indomethacin in petrolatum base in the presence of 5% of (A1) is about 0.6% of indomethacin. The release rate of (A1) from an ointment made of 1% of indomethacin, 5% (A1), and 94% petrolatum base was 4.34 $\mu g/min^{1/2}/cm^2$. Electron micrograph study on shed snake skin treated with (A1) demonstrated that (A1) interacted with the skin, making a marked morphological change in the skin.

Introduction

In a previous communication (Wong et al., 1988), we have reported the idea of design and synthesis of a new series of biodegradable transdermal penetration enhancers. The purpose of this effort is to reduce the toxicity of these compounds and at the same time to maintain their excellent transdermal penetration-enhancing effects. The general chemical structures of these compounds are given below.

In the present manuscript, we report the evaluation of the penetration-enhancing effects of these substances on the transport of indomethacin from petrolatum ointment through the shed skin of the black rat snake (*Elaphe obsoleta*). For preliminary studies, it is difficult to use human cadaver skin or animal skins such as hairless mouse

Correspondence: Ooi Wong, Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, U.S.A.



(A)

n = 0, 1 or 2

X = O or CH_2

R = CH_3 or Et

R^1 = H or CH_3

R^2 = alkyl

(A1): n = 1; $R = R^1 = CH_3$; X = CH_2; $R^2 = (CH_2)_9CH_3$

(A2): n = 0; $R = R^1 = CH_3$; X = CH_2; $R^2 = (CH_2)_{11}CH_3$

(A3): n = 2; $R = CH_3$; R^1 = H; X = CH_2; $R^2 = (CH_2)_9CH_3$

Scheme 1.

skin as models because of difficulties in obtaining adequate supplies at a reasonable cost. As a substitute for human and animal skin, Higuchi and Konishi (1987) and Ibuki (1985) have investigated the use of shed snake skin for transdermal penetration studies. It was demonstrated that shed skin of the black rat snake is suitable for this type of study. The advantages of using snake skin as a model of the stratum corneum have been demonstrated by Higuchi and Konishi (1987); we, therefore, have adopted this technique in our evaluation of the biodegradable enhancers.

Since biological membranes normally produce a large variation in penetration results, it is advantageous to use a standard for comparing the new penetration enhancers. In this regard, Azone is a good standard because it produces good penetration enhancement for a variety of drugs and has been studied extensively.

Among the compounds evaluated in this work, three compounds show penetration enhancement of indomethacin through snake skin at least equal to or better than Azone. A preliminary toxicity study on (A2) indicates that the compound has very low toxicity. The biodegradability of the enhancers has been demonstrated by hydrolysis of (A3) with porcine esterase.

Materials and Methods

Treatment of snake skin

A whole shed snake skin from a single snake was cut randomly into squares of about 4 × 4 cm and the head and tail part were discarded. Each piece of skin was hydrated overnight by first soaking and washing the skin in water (about 5–10 min) until it could be stretched and then placing it on a plastic pan in which a few drops of water were placed. The pan was floated on a covered water-bath at 32 °C overnight.

Treatment of hairless mouse skin

Eleven-week-old male hairless mice (25–30 g) were sacrificed and their dorsal and abdominal skins were removed, soaked in an isotonic phosphate buffer pH 7.2 and defatted. The freshly excised skins were then used for the penetration study.

Preparation of ointment

The ointments were prepared by mixing 1% of indomethacin, 5% of enhancer and 94% of petrolatum at 55–60 °C to give a homogeneous suspension of indomethacin. When using low-melting enhancers, indomethacin and the enhancer were first mixed well and then incorporated into the petrolatum. The mixture was then heated and stirred at 55–60 °C to give a homogeneous ointment. Indomethacin particles could be seen in all the ointments.

Penetration study and analysis of indomethacin

An ointment sample of 30 mg was applied evenly in a circular area with a diameter of 15 mm to the skin which was then mounted on the top of the receptor cell of a vertical diffusion cell assembly (modified Franz cell), Fig. 1. The cell was

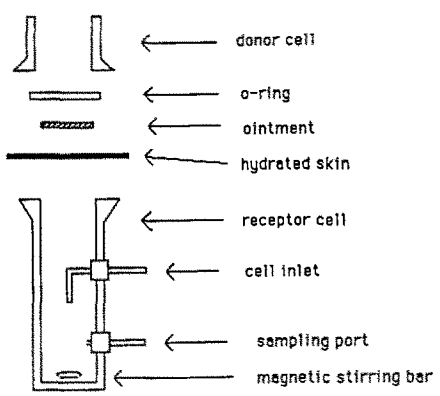


Fig. 1. Modified Franz diffusion cell used in the present shed snake skin penetration studies.

filled with an isotonic phosphate buffer of pH 7.2 with an ionic strength of 0.15 M and equipped with a small magnetic stirring bar. A rubber o-ring was placed on top of the skin followed by the donor cell. The two cells were clamped together and transferred to a water-bath (32°C) underneath which were placed several magnetic stirrers to stir the contents of the diffusion cells. Samples were withdrawn periodically by gravity. Fresh isotonic buffer was added to the cells through the cell inlets as the samples were withdrawn. The first 5 drops were discarded and the following 4 drops were collected for analysis. These 9 drops of sample solution were equivalent to 0.20 ml.

The samples were analysed for the concentration of indomethacin using either a Spectroflow 783 programmable absorbance detector with a Bio-rad HPLC pump model 1330 or a Perkin-Elmer ISS-100 HPLC apparatus with a Perkin-Elmer LC90UV spectrophotometric detector, a Perkin-Elmer ISS-100 automatic sampler and a Perkin-Elmer LCI-100 integrator with the detection wavelength set at 260 nm. A reverse phase column, RP-8 Spheri-5, 4.6 × 100 nm and a guard column OD-GU (both purchased from Brownlee Labs), were used in conjunction with the HPLC system. The flow rate was 1.0 ml/min and the solvent system was a mixture of acetonitrile (50%) and 0.01 M phosphate buffer (pH 3.0) (50%).

The concentration of indomethacin in each sample was determined from a standard curve which was obtained by plotting the peak areas obtained from the HPLC chromatograms vs 4 different standard solutions of indomethacin. The standard curves were linear over a range of concentration from 2.5 to 10 μg/ml. The receptor cell volume was calibrated by weighing the amount of water in the cell. The first sample needs no concentration correction. However, the concentration in the subsequent samples can be corrected by the following equation

$$X = X_i + \left(\frac{\text{volume withdrawn}}{\text{volume of cell}} \times X_{i-1} \right) + Y_{i-2}$$

where X = number of μg of indomethacin and X_i = number of μg of indomethacin in the receptor cell at i^{th} time, X_{i-1} = number of μg of indomethacin at $i-1^{\text{th}}$ time and Y_{i-2} = sum of concentration corrections from the previous $(i-2)$ samples.

Toxicity studies on (A2)

Acute toxicity. (A2) was dispersed in saline (0.85% NaCl in boiled deionized water) with 2% Tween 80 (Sigma). Three doses, 100 mg/kg, 320 mg/kg and 1000 mg/kg, were injected s.c. into 3 groups of Swiss white mice with 5 mice for each group. The weights of the mice ranged from 26 to 30 g. The control mice were injected with an equivalent volume of saline solution. The mice were observed periodically for 7 days and did not show any obvious abnormality. Then the mice injected with the highest dose of (A2) and the control mice were sacrificed, and dissection of the mice showed that there was no obvious damage to the internal organs nor to the tissue at the injection site.

Chronic toxicity. The preparation of sample dispersion of (A2) was the same as that in the acute toxicity experiment. Six Swiss white mice were injected s.c. at a dose of 1000 mg/kg daily for 14 days. The 6 control mice were injected with an equivalent volume of the 2% Tween 80 solution. To avoid serious contamination, a fresh (A2) suspension was prepared every two days. All mice survived after 15 days and then two mice injected with (A2) and one from the control group were sacrificed and dissected for examination. There was no obvious damage to the tissue and the internal organs of the 3 mice, but a substantial amount of yellowish material was found on the tissue below the injection site of the mice injected with (A2). The mice were kept for a period of 39 days during which one mouse was sacrificed to check for the presence of the yellowish material; it disappeared within 39 days.

Chemical stability of (A3) at pH 7.2

A preliminary stability study of (A3) (1.03×10^{-4} M) was conducted by stirring the enhancer in a solution of isotonic phosphate buffer, pH 7.2, $I = 0.15$ M at 32°C. The disappearance of the enhancer was followed by HPLC analysis. The chromatograms were obtained at 220 nm by using a Spectroflow 783 absorbance detector in conjunc-

tion with a Bio-Rad HPLC pump model 1330 and a reverse phase column, RP-8 Spheri-5, 4.6 × 100 mm and a guard column, OD-GU. The flow rate was 4.0 ml/min. The solvent system was acetonitrile (80%) and water (20%). The t_R for (A3) under this HPLC condition was 1.7 min, and a plot of the log of the peak height of (A3) vs time gave the pseudo-first-order hydrolysis rate of 0.00384 min^{-1} ($t_{1/2} = 3$ h).

Esterase hydrolysis

Porcine esterase (obtained from Sigma), diluted 1000 times with the isotonic phosphate buffer, was employed to hydrolyse (A3) (1.03×10^{-4} M). The enhancer solution was prepared by dissolving about 12 mg of the enhancer in 10.0 ml of acetonitrile to make a concentration of about 10^{-3} M. A volume of 80 μl of this solution was diluted to 2.90 ml with isotonic phosphate buffer which was pre-thermostated at 32° C. The solution was then thermostated in a water-bath at 32° C followed by the addition of 100 μl of the esterase solution equivalent to 0.30 unit of esterase. The concentration of the enhancer in the sample solution was monitored by the HPLC analytical procedure described in the above section. The kinetic runs were done in duplicate. The pseudo-first-order rate, $k_{obs} = 0.0952 \pm 0.0001$ min^{-1} with $t_{1/2} = 7.28$ min, was obtained from a plot of the log of the peak height of (A3) vs time.

Release of indomethacin and (A1) from petrolatum ointments

The release of indomethacin from petrolatum ointments with a variable concentration of indomethacin (0.1, 0.2, 0.4, 0.6, and 1.0%), 5% of (A1), and appropriate amount of petrolatum base was followed by HPLC analysis. Fig. 2 shows the illustration of the apparatus used in the release study. A sample of 100 mg of ointment was applied evenly on the external bottom of a screw cap vial with a diameter of 2.5 cm. A volume of 20.0 ml of the phosphate buffer (pH 7.2) used in the penetration study was transferred into the water-jacketed beaker which was thermostated at 32° C by a Hetofrig cooling water-bath type CB 60 manufactured by HETO, Denmark. The buffer was stirred with a small magnetic bar at a constant speed used for all the release experiments, and the vial with ointment was immersed in the buffer at a fixed distance from the bottom of the beaker. A volume of 20 μl of the sample solution was injected into the HPLC apparatus at time intervals for analysis of the amount of indomethacin released. The HPLC system used in the analysis has been described in the above section. The solvent system was a mixture of acetonitrile (50%) and 0.01 M phosphate buffer (pH 3) (50%), and the flow rate was 2.5 ml/min. The detection wavelength was set at 260 nm. The release of (A1) from a petrolatum ointment consisting of 1% of indomethacin, 5% of (A1), and 94% of petrolatum base was determined by the same analytical procedure described for the release of indomethacin except that the HPLC solvent system was acetonitrile (80%) and water (20%); the flowrate was 2.5 ml/min, and the wavelength was set at 220 nm. The t_R for (A1) was 3.53 min.

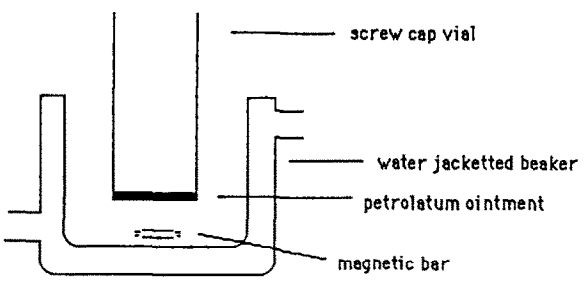


Fig. 2. Diagrammatic illustration of the apparatus used for indomethacin and (A1) release study.

Electron micrograph study

Two pieces of snake skin from a single snake were hydrated overnight in the normal way. Then a skin was treated with 5 mg of (A1) by spreading the material over a circular area with a diameter of 1.5 cm. The untreated skin was used as the control. The two skins were hydrated again at 32° C overnight. The samples were then sent to the Electron Micrograph Laboratory of the University of Kansas for electron scanning and light transmission micrograph. For the electron scanning micrograph, the specimens were cut with scissors and coated with platinum. The specimens for the light transmission micrograph were fixed

with 2% of glutaraldehyde in a buffer solution of 0.1 M of sodium cacodylate (pH 7.3).

Results and Discussion

Biodegradability of the (A) series enhancers

The (A) series enhancers are designed to be biodegradable, and it is important to demonstrate at least with in vitro experiments that they can be fragmented by enzymes such as esterases. (A3) was chosen as the model compound for this study because it bears no chiral center in its structure. (A1) and (A2) both have a chiral center which may complicate the study by producing two hydrolysis rates due to their optical isomers. The reduction of intensity of peak height (A3) versus time was obtained in the presence of 0.30 unit of porcine esterase in an isotonic phosphate buffer (pH 7.2 and $I = 0.15$ M) at 32°C. Since the enhancer shows low absorption in the UV region, the detection wavelength was set at 220 nm. The plot of logarithms of the peak heights against time indicates clearly that the esterase hydrolysis rate is pseudo-first-order for (A3), as shown in Fig. 3, with $k_{obs} = 0.0952\ min^{-1}$ and $t_{1/2} = 7.28$ min. The k_{obs} must represent contributions from hydrolysis due to the esterase and also to the isotonic phosphate buffer:

$$k_{obs} = k_{est} + k_{buf}$$

where k_{buf} is the pseudo-first-order hydrolysis rate due to the buffer solution alone, and k_{obs} is the pseudo-first-order hydrolysis rate due to the esterase and the buffer solution. The term $k_{est} = k_{enz}$ [esterase] where k_{enz} is the microscopic rate constant for esterase, and $k_{buf} = k_{buffer}$ [buffer] + $k_H + [H^+] + k_{OH^-}[OH^-] + k_0$ where k_{buffer} is the microscopic rate constant of buffer, and k_0 is the rate constant for H_2O. Since the hydrolysis rate ($0.00384\ min^{-1}$) due to the buffer term is small compared with that ($0.0952\ min^{-1}$) due to the esterase, the biodegradability of (A3) by esterase is confirmed. The hydrolysis rates, k_{obs} and k_{buf}, are comparable because the experimental conditions for determining the rates are the same (see Materials and Methods). It is not the intention of this work to investigate the detailed chemical kinetics of (A3) which may be the subject of a future study.

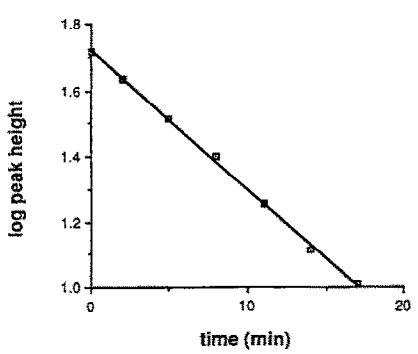


Fig. 3. A linear first-order plot of the log peak heights of (A3) vs time. $k_{obs} = 0.0951\ min^{-1}$; $t_{1/2} = 7.29$ min; $r = 0.999$.

Toxicity

The preliminary toxicity studies done in the present work were intended to see if the concept set forth previously (Wong et al., 1988) is correct, and to see how well mice tolerate (A2) given s.c. One reason for injecting (A2) subcutaneously is to allow it to be cleaved enzymatically into smaller fragments to reduce its toxicity. As the chronic toxicity results show a total of 14 g/kg (1.4% of the mice body weight) of (A2) did not cause the death of any mice. This result certainly encourages further investigation of this type of enhancer.

Penetration flux

Fick's first law states that the flux, J, of a reference material is the amount of the material passing perpendicularly through a barrier of unit area in unit time.

$$J = m/\text{area}/t$$

where m = amount of drug in μg in the receptor cell at time t. Considering the variation in characteristics of the snake skin, it would be appropriate to use the shed skin from the same snake for comparison studies, thus minimizing the variation of results due to varying snake skin characteristics. Under the same experimental conditions, we can then compare the penetration enhancement of

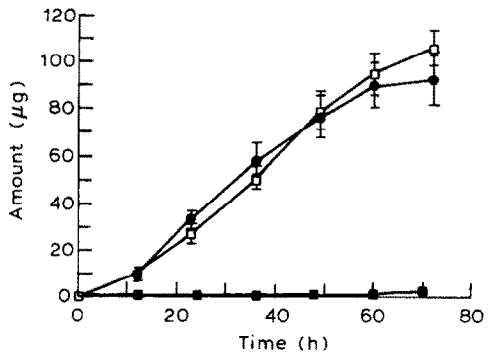


Fig. 4. Time course penetration profiles of indomethacin through snake skin at 32°C using (A2) and Azone as the enhancers. Data points represent the mean values obtained from 4 trials. The amount of indomethacin (μg) on the *y*-axis was the amount detected in the receptor cells at appropriate time. ●, 5% (A2), 1% indomethacin, 94% petrolatum; □, 5% Azone, 1% indomethacin, 94% petrolatum; ■, 1% indomethacin, 99% petrolatum.

Azone with that of the (A) series of enhancers and also with the control which has no enhancer.

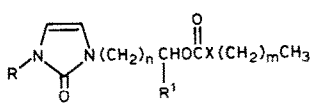
Penetration results

Since the area to which the ointments were applied was held constant as 1.77 cm^2, comparison of penetration enhancement of indomethacin by Azone or (A) series can be done by comparing the slope of the amount of indomethacin penetrating versus time. Fig. 4 shows a typical time course profile for Azone and (A2). Each data point represents a mean value obtained from 4 trials. After the first 12 h of the experiment, about 9 μg of indomethacin was detected in the receptor cells for both Azone and (A2) ointments. The S.E.M. in both cases was relatively small but increased steadily as the number of experimental hours increased. This is a typical phenomenon found in this type of snake skin penetration study. At the end of 72 h approximately 33% (100 μg) of the total applied indomethacin penetrated through the skin. In this study, the penetration fluxes of indomethacin for both Azone and (A2) are regarded as equivalent. The flux of indomethacin in the control ointments without enhancer in all experiments was always close to zero. To compare the enhancement between the new enhancers and Azone, we set a relative penetration enhancement (RPE) as follows:

$$\text{RPE} = \frac{\text{Steady state slope of an (A) enhancer}}{\text{Steady state slope of Azone}}$$

TABLE 1

Transdermal penetration enhancement of indomethacin through snake skin at 32°C by the (A) series enhancers



	R	R^1	m	n	X	RPE	m.p.	M.W.
1	Me	Me	9	1	CH_2	1.2–2.0	l.m.	338
2	Me	Me	11	1	CH_2	0.7	44.5	366
3	Me	Me	7	1	CH_2	0.5	liq	310
4	Me	Me	11	0	CH_2	0.8–1.0	43	352
5	Me	Me	9	0	CH_2	0.6	38	324
6	Me	H	9	2	CH_2	1.0	liq	338
7	Me	H	9	1	CH_2	0.6	liq	324
8	Et	Me	11	0	CH_2	0.5	42.5	366
9	Me	H	11	0	O	0.5	44	340
10	Me	H	9	0	O	0.15	40	312
11	Me	H	13	0	O	0.1	60	368
12	Me	H	7	0	CH_2	0.1	liq	282
13	Me	H	9	0	CH_2	0.1	41	310

RPE for Azone was taken as 1.0. l.m. = low melting.

By this comparison, penetration enhancement of indomethacin due to Azone is taken as 1. The results in terms of the relative penetration enhancement are shown in Table 1. Some variation of penetration enhancement has been noted when another snake skin was employed in the experiments (see Table 1), with the RPE being 1.2–2.0 and 0.8–1.0 for (A1) and (A2), respectively.

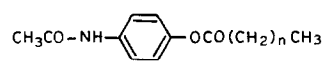
Structure–activity relationship

The transdermal penetration enhancement by the (A) series enhancers depends on the stability of the enhancers. 1-Methyl-4-imidazoline-3-methylenepivalate was found to decompose in air after a week (Wong et al., 1988). The decanoate derivative also deteriorated after a period of several months, and this may account for the low penetration enhancement (see Table 1). These possible ways to stabilize the molecules include: (1) making carbonate derivatives, compounds 9, 10 and 11 (Table 1); (2) placing a methyl group on the 3-methylene group to reduce hydrolysis, compounds 4 and 5; and (3) to elongate the methylene group to ethylene or propylene group, compounds 1, 2, 3, 6, and 7 (Table 1).

The carbonate derivatives did not produce good penetration enhancement. For example, the dodecyl derivative, compound 9, enhances penetration about 0.5 times that of Azone. This is the maximum effect of the carbonate series. This moderate increase in enhancement over the corresponding ester analogues may be due to the more stable carbonate functional group. However, the higher polar carbonate group may at the same time dampen the activity of the enhancer. (A2), having an alpha methyl group at the 3-position of the imidazolin-2-one moiety, produces about the same penetration enhancement as Azone, and is a large improvement over the methylene derivatives, compounds 12 and 13, Table 1. Also, this increment of enhancement over the carbonate series can not be due to melting point and molecular weights since these two properties of the two mini series of compounds are similar, see Table 1. By elongating the 3-methylene group, we see a steady increase in penetration enhancement from methylene (0.10), compound 13, to ethylene (0.60), compound 7, and propylene derivative (1.00), compound 6 (A3). This increment of enhancement may be attributed to the increase in both the chemical and enzymatic stability of (A3). It has been shown that the esterase stability of an ester group increases as its distance from a tertiary amino group increases (Bodor, 1984). Esterase activity in snake skin is high (Higuchi and Konishi, 1987; Nghiem and Higuchi, 1987), and the esterase stability of the enhancer may become a very important factor during the penetration processes to account for the higher penetration enhancement of (A3). At this point it would be advantageous to make the 3-isopropylene dodecanoate derivatives to see if they produce any further increase in enhancement. Indeed, (A1), compound 1, the best of all compounds shows enhancement as high as 2.00 times that of Azone at an early stage of the penetration study.

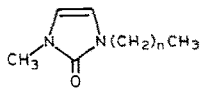
A small change in structure could result in a dramatic change in enhancement. Comparing (A2) and compound 8, we see a 0.50 time drop in enhancement by extending the 1-methyl to 1-ethyl group, although the two compounds have similar melting points. Other compounds having chemical structures similar to the (A) series by expanding the cyclic urea ring to the 6-membered ring of piperazine-2,3-diones have been prepared and tested for penetration enhancement of indomethacin, but their enhancements were about 0.1 times that of Azone (Ooi Wong, unpublished results).

Penetration enhancement of indomethacin through snake skin has been shown to depend on the lipophilicity of the enhancer. When *n*-alkanols with chain numbers ranging from 4 to 16 carbons were used as enhancers (Tsuzuki et al., 1988), the profile of penetration enhancement versus lipophilicity of enhancer is a parabolic relationship with lauryl alcohol and tridecanol exhibiting the maximum effect. This type of profile was also seen in the penetration of a series of acetaminophen prodrugs through snake skin (Nghiem et al., 1987),



and in the penetration enhancement of indomethacin by a series of cyclic ureas with struc-

tures similar to the (A) series enhancers (Ibuki, 1985). In the present work, we noted similar pro-



files for the new enhancers we developed. Table 1 shows the RPE of all new compounds. Within each mini series such as carbonate and (A1) series there is a lead or optimum compound showing the highest enhancement. The dependency of penetration enhancement on lipophilicity in the above examples is empirical but its intrinsic explanation may be related to a mixture of factors such as interaction with the skin barrier.

Hairless mouse skin penetration

Table 2 shows the results of indomethacin penetration through hairless mouse skin (Fig. 5) at 32° C using either (A1) or Azone as the enhancer. The penetration parameters are obtained from the mean values of at least 3 trials and the experimental conditions were the same as those reported for the snake skin studies discussed earlier in this communication. The slopes were obtained by linear plots of the steady state of the time course profiles (Fig. 5). The intercepts are extrapolated values from the linear plots when penetration times are zero hour and the lag times were calculated values obtained from the analytical expression of the steady state slopes when the amount of indomethacin penetrated the skins was zero. The relative enhancements were calculated by taking the control slope as one.

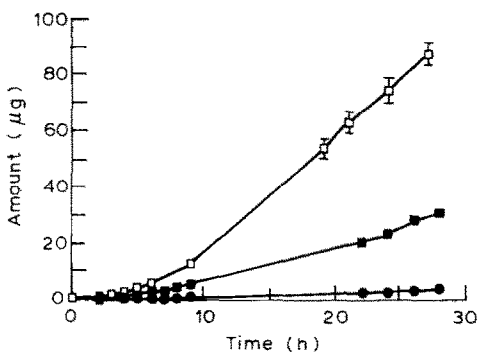


Fig. 5. Time course penetration profiles of indomethacin through hairless mouse dorsal skin at 32° C using (A1) and Azone as the enhancers. □, 5% (A1), 1% indomethacin, 94% petrolatum; ■, 5% Azone, 1% indomethacin, 94% petrolatum; ●, 1% indomethacin, 99% petrolatum.

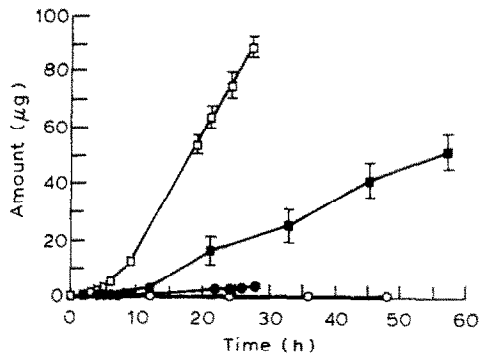


Fig. 6. Comparison of permeability of snake skin and hairless mouse dorsal skin by indomethacin using (A1) as the enhancer at 32° C. □, hairless mouse skin, 5% (A1), 1% indomethacin, 94% petrolatum; steady-state flux = 1.933 μg/h/cm^2; ●, hairless mouse skin, 1% indomethacin, 99% petrolatum; steady-state flux = 0.0922 μg/h/cm^2; ■, snake skin, 5% (A1), 1% indomethacin, 94% petrolatum; steady-state flux = 0.606 μg/h/cm^2; ○, snake skin, 1% indomethacin, 99% petrolatum; steady-state flux is close to zero.

The penetration enhancement of indomethacin by Azone is 8 times better than the control, however, that by (A1) is 26 times. This means that the enhancement by (A1) over Azone is 3.2 times. Nevertheless, when shed snake skin was used as the model membrane, this factor was reduced to less than 2 (see Table 1). This is possibly due to the difference in characteristics and resistance of the two types of skins. This type of phenomenon has also been noted by Okamoto et al. (1987) in their study of enhanced penetration of mitomycin C through hairless mouse and rat skin by several new enhancers. The difference in penetration rates of indomethacin through hairless mouse skin and snake skin is demonstrated in Fig. 6 where the flux of indomethacin through snake skin for the control (0.0091 μg/h/cm^2) is close to zero. There is a significant increase in flux of indomethacin compared with control (0.0922 μg/h/cm^2) when the skin barrier was replaced with hairless mouse skin. This indicates that hairless mouse skin is more permeable than the snake skin used in the present work. It is interesting to note that the dorsal and abdominal skins of hairless mice pro-

TABLE 2

Effects of (A1) and Azone on indomethacin penetration through dorsal hairless mouse skin at 32 °C

	n	S.S. lag time	S.S. slope	Intercept	*r*	RE	S.S. flux
Control	4	5.7 (0.77)	0.163 (0.0257)	−0.922	0.993	1	0.0922
Azone	4	4.9 (0.62)	1.305 (0.0383)	−6.42	0.995	8	0.737
(A1) [a]	3	5.9 (0.49)	4.227 (0.336)	−25.5	1.000	25.9	2.388
(A1) [b]	3	5.8 (0.052)	3.421 (0.642)	−19.9	0.998	21.0	1.933

S.S., steady state, S.S. lag time in h; S.S. slope in $\mu g/h/1.77\ cm^2$; S.S. flux in $\mu g/h/cm^2$; S.D. in parentheses.

S.D. $= \sqrt{\frac{\Sigma(x_i - x)^2}{N-1}}$ where x_i = individual value; x = mean value; N = the number of degree of freedom.

r = correlation coefficients of the regression line; RE = enhancement by taking the control as 1.

[a] Dorsal skin.

[b] Abdominal skin.

duce significantly different fluxes of indomethacin at the later stage of the penetration study (see Table 2). This may be due to the difference in thickness of the two skins. The complexity of human and other animal skins have been well documented in the literature (Simon et al., 1978). The variation in permeability between human cadaver and hairless mouse skin can be seen by comparing the fluxes of clonidine through hairless mouse skin ($0.180\ \mu g/h/cm^2$) with that through the human cadaver skin ($0.127\ \mu g/h/cm^2$) (Chien, 1987).

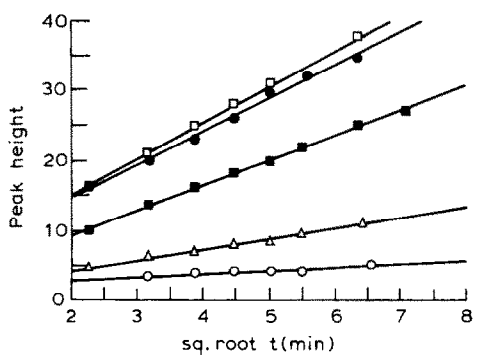


Fig. 7. Release profiles of indomethacin for petrolatum ointments consisting of variable indomethacin concentration, 5% of (A1), and appropriate amount of petrolatum base. Standard peak height = 11.8 division/μg/ml. ○, 0.1% indomethacin; release rate = 0.17 $\mu g/min^{1/2}/cm^2$; △, 0.2% indomethacin; release rate = 0.53 $\mu g/min^{1/2}/cm^2$; ■, 0.4% indomethacin; release rate = 1.23 $\mu g/min^{1/2}/cm^2$ ●, 0.6% indomethacin; release rate = 1.66 $\mu g/min^{1/2}/cm^2$; □, 1.0% indomethacin; release rate = 1.79 $\mu g/min^{1/2}/cm^2$.

Release of indomethacin and (A1) from petrolatum ointments

The release of both indomethacin and (A1) from the petrolatum ointments followed the Higuchi equation of drug release for drug suspended in an ointment base (Higuchi, 1961)

$$Q = [D(2A - C_s)C_s t]^{1/2}$$

where Q is the amount of drug depleted from the ointment at time t, D is the diffusion coeffi-

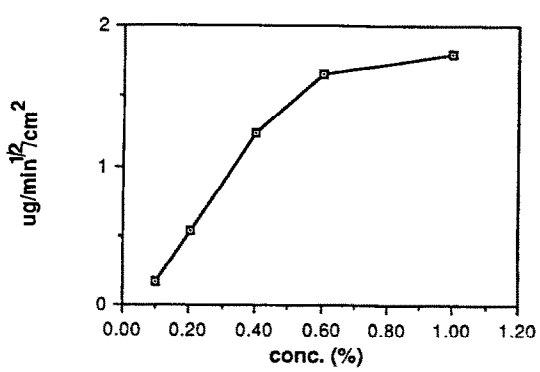


Fig. 8. Plot of release rates of indomethacin vs concentration of indomethacin in ointments.

cient of the drug in that particular ointment, A is the total amount of drug incorporated into the ointment, and C_s is the saturated solubilized concentration of the drug in the ointment. As long as $A \gg C_s$, a linear relationship can be obtained by plotting Q versus $t^{1/2}$. It is very difficult to measure the saturation concentration of indomethacin in petrolatum base using ordinary methods. Therefore, we adopted a more practical way to make use of the Higuchi equation by determining the release rates of a series of ointments consisting of a variable concentration of indomethacin and 5% of (A1). The release rate should increase as the concentration of solubilized indomethacin increase until a saturation point was attained, and beyond such point the excess drug would be present in a suspended form in the petrolatum base. This is what we observed in the release study, see Fig. 7. By plotting the release rates of indomethacin against the concentration (%) of indomethacin incorporated in the ointments, the release rate increases linearly as the indomethacin concentration increases, and levels at 0.6%, Fig. 8. The saturation point is about 0.6% of indomethacin in the petrolatum ointments. Thus, at a 1% level of indomethacin in the petrolatum ointments used in the skin penetration studies, it is anticipated that much of the indomethacin was suspended in the ointments.

It is very important to demonstrate that (A1) was released from the ointments since it can not interact with the skin if it stays in the ointments.

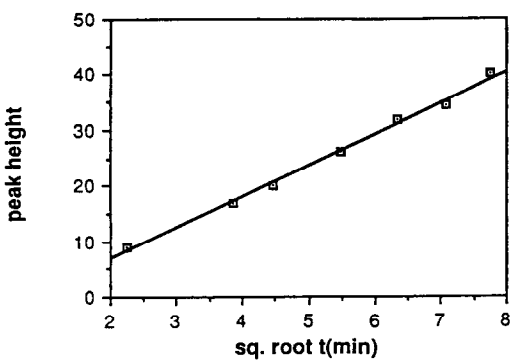


Fig. 9. Release of (A1) from petrolatum ointment consisting of 1% indomethacin, 5% of (A1), and 94% of petrolatum base. Standard peak height = 5.27 division/μg/ml; release rate = 4.34 $\mu g/min^{1/2}/cm^2$.

Fig. 9 shows the release graph of (A1) from ointments consisting of 1% of indomethacin, 5% of (A1), and 94% petrolatum base. After 60 minutes, 3.0% of (A1) was released from the ointment at a rate of 4.34 $\mu g/min^{1/2}/cm^2$. At this release rate over a period of 70 h, we believe that (A1) has an effect on the skin membrane.

Electron micrograph study

To confirm our hypothesis that the enhancers interacted with the skin, we carried out an electron micrograph study to gain physical evidence of the morphological changes in the skin caused by enhancer. The shed snake skin of *Elaphe obsoleta* has 3 different layers constituted mainly of keratin and lipids. The outermost layer is called β-layer (β-keratin rich), the middle layer is called mesos layer (α-keratin and lipid rich), and the innermost layer is called α-layer (α-keratin rich) (Ibuki, 1985). The mesos layer has been claimed to be similar to the human stratum corneum (Ibuki, 1985). By treating the snake skin with (A1), we would expect a morphological change in the skin if there is interaction of the skin with (A1). Fig. 10 shows the scanning electron and light transmission micrographic pictures of shed snake skin for control, and skin treated with (A1). A remarkable morphological change on the surface between the scales of the shed snake skin developed after the skin was treated with (A1), see Fig. 10a, b. No significant morphological change was observed on the surface of the scales. The change on the skin surface indicates that (A1) has altered the skin material by some kind of interaction. This is confirmed by the light transmission micrograph of the cross-section of the skins, Fig. 10c, d. (A1) has swollen the 3 layers, α, β and mesos, indicating that the enhancer has strong interaction with both keratins and lipids. This study confirms the role of our new enhancers includes a reduction of skin permeability. A similar study was conducted by using Azone as the enhancer and showed that the micrographic picture of the snake skin treated with Azone was similar to that of the control (Wong et al., 1989).

Possible mechanism of enhancers

The electron micrograph study, discussed in the above section, clearly shows that (A1) interacted

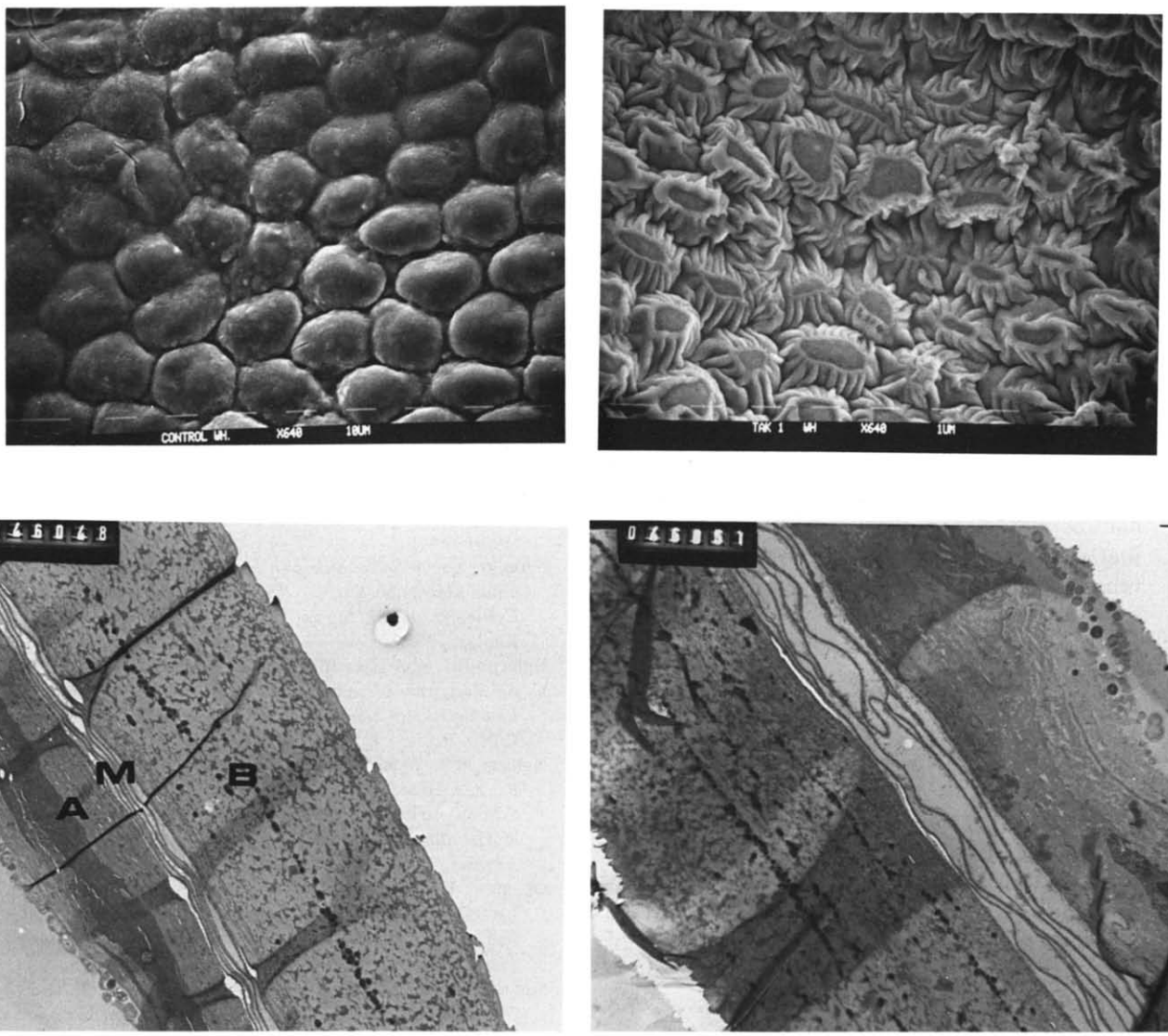


Fig. 10. a: scanning electron photomicrograph of the surface of shed snake skin (*Elaphe obsoleta*). Control. ×450. b: scanning electron photomicrograph of the surface of shed snake skin (*Elaphe obsoleta*) treated with (A1). ×450. c: light transmission photomicrograph of the cross-section of shed snake skin (*Elaphe obsoleta*). Control. ×2150. A, α-layer; M, mesos layer; B, β-layer. d: light transmission photomicrograph of the cross-section of shed snake skin (*Elaphe obsoleta*) treated with (A1). ×2000. Darker spots are due to folding of the specimens.

with the snake skin and changed the morphology of the skin. (A1) swelled the keratin-rich layers, and, particularly, the lipid-rich mesos layer, thus reducing the density of the barrier. This will cause a loosening effect on the skin. However, enhancers do not always work in this way. We have developed another series of excellent enhancers which fused the lipid-rich mesos layer instead of swelling it (Wong et al., 1989).

The release of indomethacin from ointments is expected to be under sink conditions. From this study, it is estimated that the saturated concentration of indomethacin in petrolatum base is about 0.6% of indomethacin, and, therefore, at a 1% level

of indomethacin, the thermodynamic activity of indomethacin in petrolatum is expected to be constant, producing a constant release rate for indomethacin. The release rate of indomethacin from ointment made of 1% indomethacin, 5% (A1), and 94% petrolatum base was 13.6 $\mu g/h/cm^2$, see Figs. 7 and 8. Comparing the release rate with the steady-state fluxes of indomethacin, using the same ointment, obtained from the penetration studies for snake skin (0.606 $\mu g/h/cm^2$), and for hairless mouse skin (1.93 $\mu g/h/cm^2$), see Fig. 6, we can suggest that penetration of the skin barriers is the rate-limiting step. This information further supports the hypothesis that the role of the new enhancers includes an increase in skin permeability. Strong evidence for supporting the hypothesis of increase in skin permeability comes from the hairless mouse skin penetration study. The fact that the enhancement by (A1) over Azone is 3.2 times using hairless mouse skin as the model membrane while it is less than 2 times when shed snake skin was used in the study, strongly suggests that the difference in penetration fluxes of indomethacin is due to the interaction of enhancers with the skin barrier. The release rate of indomethacin for the ointment, made of 1% of indomethacin, 5% of (A1), and 94% of petrolatum base, is expected to be the same for either hairless mouse skin or snake skin penetration study since there are films of water between the ointment and the skins. This is also true in the case where ointment was made by replacing (A1) with 5% of Azone.

The evidence presented in this paper shows that the skin is the rate-limiting barrier for the penetration of indomethacin through the skin. Therefore, the observed effect of the penetration enhancer on penetration rate should affect the rate controlling skin barrier, an increase in the diffusion coefficient and/or drug solubility in the stratum corneum-like phase in the snake skin.

Acknowledgements

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