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Research Article

New Alkyl *N,N*-Dialkyl-Substituted Amino Acetates as Transdermal Penetration Enhancers[1]

Ooi Wong,[2] James Huntington,[2] Toshiaki Nishihata,[2] and J. Howard Rytting[2,3]

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New alcohol derivatives of *N,N*-disubstituted amino acids with a low toxicity have been synthesized and evaluated for their transdermal penetration enhancing effects on the transport of indomethacin from petrolatum ointments across shed skin of black rat snake (*Elaphe obsoleta*). The derivatives show excellent penetration enhancement of indomethacin, as high as 3.8 times that of Azone, with decyl *N,N*-dimethylamino acetate as the lead compound in the series. The release of indomethacin from an ointment containing 1% indomethacin, 5% dodecyl *N,N*-dimethylamino acetate, and 94% petrolatum was 3.15 $\mu g/min^{1/2}/cm^2$. Saturation studies performed by incorporating varying concentrations of indomethacin, from 0.1 to 10%, into the ointments and determination of the fluxes of indomethacin demonstrated that the saturated concentration of indomethacin in petrolatum base was approximately 1%. Penetration fluxes of indomethacin (1%) through snake skin increased linearly as the concentration of dodecyl *N,N*-dimethylamino acetate increased from 2.5 to 15%. Experiments involving the pretreatment of the snake skins with dodecyl *N,N*-dimethylamino acetate indicated that pretreatment of the skin increased the skin permeability significantly. Electron micrograph studies on the snake skin treated with dodecyl *N,N*-dimethylamino acetate show clearly that the enhancer interacted with both the lipid-rich layer (mesos phase) and the keratin-rich layers (both alpha and beta phases).

KEY WORDS: penetration enhancers; indomethacin; Azone; percutaneous absorption; snake skin.

INTRODUCTION

Many drugs of varying molecular size require penetration enhancers for transport across skin or mucous membranes. A number of problems and strategies for delivery of macromolecules have been documented in the literature (1,2). The use of penetration enhancers has been reviewed by Vaidyanathan *et al.* (3), Cooper and Berner (4), and Lee (2), but more effective penetration enhancers of a low toxicity need to be developed. Work in our laboratory focuses on biodegradable penetration enhancers using shed snake skin (*Elaphe obsoleta*) as the skin model (5–7). The rationale and justification for using snake skins in transdermal penetration study have been addressed by Higuchi and Konishi (8). Some of the advantages cited include the following: (a) there is a similarity between snake skin and human stratum corneum, (b) shed snake skin can be obtained with no injury and without killing the animal, (c) there is less variation in permeation results, (d) snake skin can be kept for months under refrigeration without deterioration, (e) snake skin is less expensive, and (f) snake skin lacks hair follicles. One approach to reducing toxicity involves biodegradable compounds which make use of the enzyme activity in the skin to fragment the penetration enhancers into smaller innocuous compounds. Several biodegradable unsaturated cyclic urea derivatives show penetration enhancement of indomethacin at least equal to that of Azone (7), but it may be possible to achieve a greater enhancing effect: Further, the slow onset of action of most enhancers should be overcome to widen their utility. The aim of the present work was to develop potent, fast-acting nontoxic transdermal penetration enhancers.

Some recently developed enhancers were claimed to have good enhancing effects, namely, macrocycles (9), novel cyclic ureas (10), and enhancers with terpene moieties (11). Azone was patented by Nelson Research and Development Co. in 1983 and has since been studied extensively as a new type of penetration enhancer, but it may cause damage to skin after prolonged exposure. The safety of Azone has been studied by Stoughton and McClure (12) and by Stoughton (13). A recent study on percutaneous absorption and elimination of Azone in humans by Wichers *et al.* (14) indicates that Azone is safe for human use. However, long-term chronic toxicity data have not been reported, and the median lethal dose (LD_{50}) of Azone (232 mg/kg) given i.p. to mice (15) may discourage its practical use.

Long-chain alcohols are known to enhance the trans-

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[2] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66045.

[3] To whom correspondence should be addressed.

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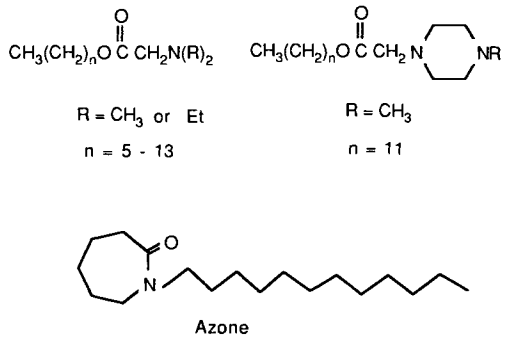
dermal penetration of indomethacin (16); therefore, an ester or other functional derivatives of alcohols might produce good enhancing effects. Amino acid esters of the long-straight chain alcohols were synthesized in the present work because they can be cleaved by esterases in the skin into alcohols and substituted amino acids. In the present work, we have prepared some new alkyl *N,N*-disubstituted acetates and evaluated the transport of indomethacin across shed snake skin. Using Azone as a standard of comparison, it was found that dodecyl *N,N*-dimethylamino acetate, decyl *N,N*-dimethylamino acetate, and octyl *N,N*-dimethylamino acetate produce relative enhancements of 2.0, 3.8, and 2.5 times of Azone, respectively, at the early stages of the penetration experiments.

MATERIALS AND METHODS

General Structures

The general structures of the new enhancers synthesized in the present work and the structure of Azone are given in Scheme I.



Scheme 1

Chemistry

The synthesis of the long-chain alkyl *N,N*-dimethylamino acetates involves two steps. The first step is the preparation of alkyl chloroacetates by reaction of *n*-alkanols with chloromethyl chloroformate in chloroform using triethylamine as the base. The second step is the condensation of the long-chain alkyl chloroacetates with excess disubstituted amines (excess amine can act as the base). The fact that these enhancers can be cleaved by the esterases in the skin indicates that a second enhancement effect may be developed by the fragments of the enhancers, the corresponding alcohols, and the disubstituted amino acids.

Synthesis

Reagents and Solvents

Chloroform (Fisher Scientific), triethylamine (Sigma), *n*-alkanols (Aldrich), chloromethyl formate (Aldrich), dimethylamine (0.1 *M* in diethyl ether; Alfa), diethylamine (Aldrich), 1-methylpiperazine (Aldrich), diethyl ether (Fisher Scientific), and ethyl acetate were used without further treatment. Azone was obtained from Nelson Research and Development Co.

Apparatus and Structure Characterization

The compounds synthesized in this work were characterized by infrared, proton nuclear magnetic resonance and mass spectroscopy. The purity of the new alkyl *N,N*-disubstituted amino acetates was checked by thin-layer chromatography (TLC) using ethyl acetate as the developing solvent system and iodine vapor as the visualizing agent. The enhancers were purified using silica gel as the column supporting material and ethyl acetate as the eluent. Proton nuclear magnetic resonance spectra were recorded on a Varian T-60 spectrometer using carbon tetrachloride (CCl_4) as the solvent and tetramethyl silane as the internal standard. Infrared spectra were recorded on a Beckman Accu-Lab 2 spectrophotometer, and mass spectra on a NERMAG R-10-10 Quadrupole mass spectrometer. Thin-layer chromatograms were obtained by using Analtech silica gel TLC plates (HPTLC-GHLF) and ethyl acetate as the solvent system.

Dodecyl Chloroacetate

A mixture of dodecanol (10 g, 0.054 mol), chloromethyl chloroformate (6 g, 0.05 mol), and triethylamine (5.5 g) in 40 ml of dry chloroform was stirred at room temperature for 16 hr. After washing the reaction mixture two times with water (50 ml each washing), the organic phase was dried over anhydrous magnesium sulfate. Removal of the solvent yielded 13.3 g of liquid product (94%) which was chromatographed through a column of florosil using chloroform as the eluent. ^1H NMR (CCl_4): δ 0.90 (3H, t, $CH_3[CH_2]_{10}CH_2O-$); 1.28 (20H, s, broad, $CH_3[CH_2]_{10}CH_2O-$); 3.39 (2H, s, $-CH_2Cl$); 4.11 (2H, t, J = 6 Hz, $CH_3[CH_2]_{10}CH_2O-$). IR (film): 2940, 2880 (CH); 1765, 1745 (C=O); 1180 cm^{-1} C–O–C).

Dodecyl N,N-*Dimethylamino Acetate*

Dodecyl chloroacetate (5.5 g, 0.021 mol) was dissolved in ether (20 ml) in a flask, and the solution cooled in an ice bath. Dimethylamine (40 ml, 0.1 *M* in ether) was then added to the cooled solution of dodecyl chloroacetate. After stirring the reaction mixture at room temperature for 2 hr, some white solid formed and was filtered through a filter paper. The filtrate was concentrated to give a liquid product which was chromatographed through a column of silica gel (60–200 mesh) using ethyl acetate as the eluent. The pure fractions were combined and the solvent was removed by a rotovap under reduced pressure giving a total of 5.8 g (100%) of dodecyl *N,N*-dimethylamino acetate. The TLC R_f value was 0.43. ^1H NMR (CCl_4): δ 0.83 (3H, t, $CH_3[CH_2]_{10}CH_2O-$); 1.20(20H, s, broad, $CH_3[CH_2]_{10}CH_2O-$); 2.23 (6H, s, $[CH_3]_2N-$); 3.00 (2H, s, $-COCH_2-N$); 3.93 (2H, t, J = 6 Hz, $CH_3[CH_2]_{10}CH_2O-$). IR (film): 2940, 2870, 2790 (CH); 1760, 1740 (C=O); 1200, 1170 cm^{-1} (C–O–C). MS 271, $C_{16}H_{33}NO_2$ requires 271.

Decyl 3-Methylpiperazine Acetate

The reaction procedure is the same as that for dodecyl *N,N*-dimethylamino acetate described in the above section. A mixture of decyl chloroacetate (5 g, 0.021 mol) and 1-methylpiperazine (2 equivalents) in ether (20 ml) was stirred for 1.5 days. The usual workup gave a liquid product (6 g,

96%) which was chromatographed through a column of silica gel using ethyl acetate as the eluent. The TLC R_f value was 0.15. ^{1}H NMR (CCl_4): δ 0.73 (3H, t, $CH_3[CH_2]_8CH_2O-$); 1.13 (16H, s, broad, $CH_3[CH_2]_8CH_2O-$); 2.03(3H, s, CH_3N-); 2.27 (8H, multiplets, $-NCH_2CH_2N-$); 2.91 (2H, s, $-CH_2N-$); 3.87(2H, t, J = 6 Hz, $CH_3[CH_2]_8CH_2O-$). IR (film): 2940, 2870, 2700 (CH); 1755 (C=O); 1170 cm^{-1} (C−O−C). MS 298, $C_{17}H_{34}N_2O_2$ requires 298.

Evaluation of the New Enhancers

Release of Indomethacin from Petrolatum Ointments

The release of indomethacin from petrolatum ointments with a variable concentration of indomethacin (0.1, 0.25, 0.5, and 1.0%) and 5% of dodecyl *N,N*-dimethylamino acetate was followed by high-performance liquid chromatographic (HPLC) analysis. Figure 1 shows an 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 rpm 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 included a Kratos Spectroflow 783 programmable absorbance detector with a Bio-Rad Model 1330 HPLC pump in conjunction with a reverse-phase column, RP-8 Spheri-5, 4.6 × 100 mm, and a guard column, OD-GU. 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 rate of indomethacin for ointment containing 0.25% indomethacin was checked by a duplicate run with 0.82 $\mu g/min^{1/2}/cm^2$.

Penetration Study

Azone and the seven alkyl *N,N*-disubstituted amino acetates prepared in the present work were used as enhancers in these penetration experiments. Azone was used as the standard for comparing penetration enhancements of the new enhancers. The experimental conditions for the control, where the ointments were prepared without enhancer, Azone and the new enhancers were identical to those of the experiments with enhancer throughout the experiments.

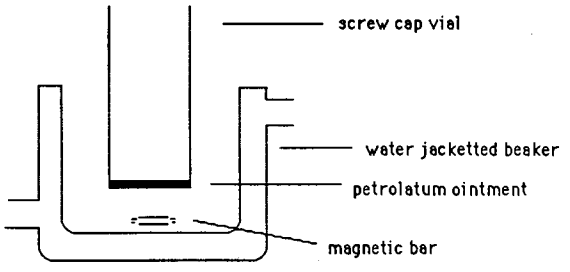


Fig. 1. Diagramatic illustration of the apparatus used for indomethacin release from an ointment.

Treatment of Snake Skins

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 to 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.

Preparation of Ointments and Penetration Experiments

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. Indomethacin particles could be seen in all the ointments.

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), with a receptor cell volume of about 10 ml. The cell was 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 five drops were discarded and the following four drops were collected for analysis. These nine drops of sample solution were equivalent to 0.20 ml.

The samples were analysed for the concentration of indomethacin using 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 mm, 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 versus four different standard solutions of indomethacin. The standard curves were linear over a range of concentrations from 2.5 to 10 μg/ml.

Saturation Study

A series of ointments containing different concentrations of indomethacin, ranging from 0.1 to 10%, 5% of dodecyl *N,N*-dimethylamino acetate, and an appropriate amount of petrolatum was prepared and studied for the pen-

etration fluxes of indomethacin in the usual way described above.

Concentration Effect

A series of ointments with 1% of indomethacin, a variable concentration of dodecyl *N,N*-dimethylamino acetate ranging from 2.5 to 15%, and an appropriate amount of petrolatum base was prepared as described above and used in the normal penetration study described previously.

Pretreatment

Eight pieces of snake skin from a single snake were hydrated overnight as described earlier. Four of the skins were then pretreated with 5 mg of dodecyl *N,N*-dimethylamino acetate by spreading the material over a circular area with a diameter of 1.5 cm. The eight skins were hydrated again overnight and used in the penetration study described in the section of saturation study.

Solution Studies

Two solution formulations were prepared by mixing the ingredients together: (i) 1% of indomethacin (40 mg), Tween 80 (Sigma) (80 mg), and 3.92 ml of phosphate buffer (pH 7.2); and (ii) 1% of indomethacin (20 mg), Tween 80 (40 mg), dodecyl *N,N*-dimethylamino acetate (100 mg), and 1.84 ml of phosphate buffer (pH 7.2). The ingredients were stirred with a small magnetic bar to give homogeneous suspensions.

Six pieces of snake skin were first hydrated as described above and then two pieces were treated with 5 mg of dodecyl *N,N*-dimethylamino acetate in the same manner as described in the section of pretreatment. All the skins were hydrated again overnight. Then two normal skins were used as the control for formulation i, and the other two normal skins were used for formulation ii. The pretreated skins were used for formulation i. The cell assemblies were clamped together and 0.5 ml of the solution formulations i and ii was transferred accordingly into the donor cells. The cell assemblies were thermostated at 32°C and a one-point sample was taken at 24 hr. Analysis of the indomethacin concentration was performed with the HPLC procedure described in the section of release study except with a flow rate of 2.0 ml/min.

Electron Micrograph Study

Three pieces of snake skin from a single snake were hydrated overnight in the normal way. Then two skins were treated separately with 5 mg of Azone and 5 mg of dodecyl *N,N*-dimethylamino acetate by spreading the material over a circular area with a diameter of 1.5 cm. The untreated skin was used as the control. The three 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 vapor. The specimens for light transmission micrograph were fixed with a buffer solution of 0.1 *M* sodium cacodylate (pH 7.3) containing 2% gluderaldehyde.

Chronic Toxicity Study on Dodecyl *N,N*-Dimethylamino Acetate

Dodecyl *N,N*-dimethylamino acetate (10%, w/w) was dispersed in saline (0.85% of NaCl in deionized boiled water) with 2% of Tween 80 (Sigma). Seven male CF-1 white mice were injected subcutaneously daily at the dorsal site with the dodecyl *N,N*-dimethylamino acetate dispersion at a dose of 1 g/kg for 7 days. Six control mice were injected daily with an equivalent volume of 2% of Tween 80 (Sigma) saline solution for 7 days.

Irritation Study on Dodecyl *N,N*-Dimethylamino Acetate

The irritation potential of dodecyl *N,N*-dimethylamino acetate was checked by spreading 25–35 mg of petrolatum ointment made of 1% indomethacin, 5% dodecyl *N,N*-dimethylamino acetate, and 94% petrolatum base on a dorsal site of a hairless mouse. Every 1 or 2 days, a dose of ointment was applied to the same dorsal site of the mouse; up to nine doses were given over a period of 12 days. This dorsal site of the hairless mouse was accessed for redness by comparison with other dorsal areas of the same mouse to which ointment had not been applied.

RESULTS AND DISCUSSION

Synthesis

Alkyl chloromethyl acetates prepared in single batches were obtained in almost quantitative yields by reaction of chloromethyl chloroformate with *n*-alkanols in the presence of triethylamine (Table I). The purification of these compounds by column chromatography is relatively easy using silica gel as the supporting material and chloroform as the eluent. The new alkyl *N,N*-dialkyl-substituted amino acetates prepared in single batches were also obtained in very high yields (Table II) by condensation of the corresponding alkyl chloromethyl acetates with excess disubstituted amines. Purification of most of these new compounds is also straightforward due to their high yields and high TLC R_f values using ethyl acetate as the developing solvent. However, the shorter-chain derivative, hexyl *N,N*-dimethylamino acetate, was more difficult to purify because the R_f value of the desired compound was close to that of the impurities. Because of the low UV absorption of these com-

Table I. Yields of Alkyl Chloromethyl Acetates[a]

$CH_3(CH_2)_nO\,C\,CH_2Cl$ (O)

No.	*N*	Yield (%)
1	13	85
2	11	94
3	9	100
4	7	100
5	5	91
6	3	61

[a] These compounds exhibit similar infrared and proton nuclear magnetic spectroscopic features.

Table II. Yields of Alkyl *N,N*-Disubstituted Amino Acetates[a]

No.	R	*N*	Yield (%)	MS[b]
		$CH_3(CH_2)_nO\,\overset{O}{\overset{\|}{C}}\,CH_2N(R)_2$		
1	Me	13	90	299
2	Me	11	100	271
3	Me	9	91	243
4	Me	7	72	215
5	Me	5	84	187
6	Et	11	95	299
		$CH_3(CH_2)_nO\,\overset{O}{\overset{\|}{C}}\,CH_2\,N\langle\quad\rangle NR$		
7	Me	9	96	298

[a] These compounds exhibit similar infrared and proton nuclear magnetic spectroscopic features.
[b] Molecular ion obtained from mass spectra for the individual compound.

Table III. Transdermal Penetration Enhancement of Indomethacin Through Shed Snake Skin at 32°C Using Alkyl *N,N*-Disubstituted Amino Acetates as the Enhancers

No.	R	*N*	Relative enhancement[a]
		$CH_3(CH_2)_nO\,\overset{O}{\overset{\|}{C}}\,CH_2N(R)_2$	
1	Me	13	1.0
2	Me	11	2.0
3	Me	9	3.8
4	Me	7	2.5
5	Me	5	1.4
6	Et	11	0.7
		$CH_3(CH_2)_nO\,\overset{O}{\overset{\|}{C}}\,CH_2\,N\langle\quad\rangle NR$	
7	Me	9	0.4

[a] The relative enhancement of indomethacin by Azone is taken as 1.0.

pounds, their purity was checked by thin-layer chromatography using ethyl acetate as the developing solvent and iodine vapor as the visualizing agent. In all cases, the compounds show a single spot on the TLC plates. Nuclear magnetic resonance, infrared, and mass spectra for the compounds exhibit similar characteristic features and are consistent with the structures assigned. It is important to mention that all the materials used to construct the target molecules are inexpensive and easily obtainable from commercial sources. Also of importance is that the synthesis of this type of new enhancer is simple with high yields of products.

Penetration Enhancement

Table III shows the transdermal penetration results of the new alkyl *N,N*-disubstituted amino acetates prepared in this work using shed snake skin as the skin model. The relative enhancements were calculated according to the following equation:

$$\text{relative enhancement} = \frac{\text{penetration enhancement by the new enhancer}}{\text{penetration enhancement by Azone}}$$

By this comparison, penetration enhancement of indomethacin due to Azone is taken as 1. All the relative enhancements shown in Table III are based on the early hours of the penetration studies and on the averages of at least three experimental runs. Figure 2 shows a typical time-course penetration profile of indomethacin using dodecyl *N,N*-dimethylamino acetate as the enhancer. The new enhancer acts quickly on penetration enhancement and exhibits a leveling tendency after 24 hr. This is probably due to the fact that most of the solubilized indomethacin in the petrolatum ointments has been released and penetrated through the skin into the receptor cells during the early hours of the experiment. The solubilized indomethacin had become exhausted at the later hours and this in turn reduced the penetration flux of indomethacin. At 24 hr, 54 μg of indomethacin was detected in the receptor cells, equivalent to 18% of the amount of indomethacin applied to the donor cells. At the end of 72 hr, 101 μg of indomethacin penetrated the skin, equivalent to 33% of the total indomethacin applied. As for Azone, 8.3% (25 μg) of the total applied indomethacin was found in the receptor cells at 24 hr, and 82 μg of indomethacin (27%) in the receptor cells at the end of 72 hr. The control ointment made of 1% indomethacin and 99% petrolatum always shows fluxes of indomethacin close to zero. Among these new enhancers, decyl *N,N*-dimethylamino acetate is the most effective derivative under the present experimental conditions, showing relative enhancement of 3.8 times that

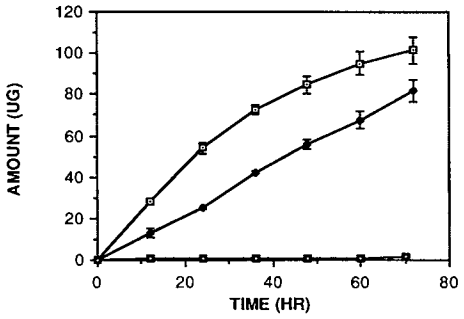


Fig. 2. Time-course penetration profiles of indomethacin through shed snake skin at 32°C using dodecyl *N,N*-dimethylamino acetate and Azone as the enhancers. The *y* axis represents the amount of indomethacin in the receptor cell at the specific time. The data points were the mean values obtained from four trials and the bars are the standard errors of the means. (□) 1% indomethacin, 5% dodecyl *N,N*-dimethylamino acetate, and 94% petrolatum. (♦) 1% indomethacin, 5% Azone, and 94% petrolatum. (■) 1% indomethacin, 99% petrolatum.

of Azone. For this derivative, 39% of the total amount of indomethacin applied can be detected in the receptor cells after 72 hr.

The fact that these new enhancers are several times more effective than Azone suggests that the time of the onset of action of these compounds is short. Rapid action is one of the most important criteria for an effective penetration enhancer. For dodecyl *N,N*-dimethylamino acetate, as much as 25 μg indomethacin could be detected in the receptor cell after 12 hr, while at the same stage 13 μg indomethacin was detected in the cells with Azone as the enhancer (Fig. 2). Because of the fast action of the enhancer, steady-state flux of indomethacin may have developed during the early hours of the experiments.

Structure–Activity Relationship

The penetration enhancement results seem to be related to the lipophilicity of the enhancers. Figure 3 shows a plot of relative enhancement versus the carbon number of the alkyl *N,N*-dimethylamino acetates. As the carbon number increases, the relative enhancement increases, reaching a maximum with the carbon number 10, beyond which the enhancement begins to fall. This phenomenon has been observed in several series of compounds. As we have noticed in the unsaturated cyclic urea series of enhancers, a slight modification in the chemical structure may result in a dramatic change in penetration enhancement (7). Again, in this new type of alkyl *N,N*-disubstituted amino acetates, we noticed the same phenomenon. By replacing the *N*-methyl groups with ethyl groups, the relative enhancement drops from 2.0 to 0.7 (Table III, compounds 2 and 6), and the piperazyl derivative, 7, has an even lower activity.

Toxicity

The preliminary chronic toxicity study on dodecyl *N,N*-dimethylamino acetate indicates that the compound has a low toxicity. All mice survived a total dosage of 7 g/kg. However, the mice lost weight after the third day of injection. They regained their normal weight during the postinjection period (Fig. 4). Tween 80 was incorporated in the sample preparation to solubilize the enhancer.

Release of Indomethacin from Petrolatum Ointments

The release of indomethacin followed the Higuchi equation of drug release for at least 10% of the total indomethacin incorporated:

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

where Q is the amount of drug depleted from the ointment at time t, D is the diffusion coefficient 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. By plotting the peak heights of the HPLC chromatograms versus the square roots of time, a linear graph was obtained with a slope having units of peak height/min$^{1/2}$. The slopes obtained in this way may be converted into μg/min$^{1/2}$/cm^2 (see Table IV) by the following equation:

$$\frac{\text{slope (PH/min}^{1/2})}{\text{standard solution (PH/μg/ml)}} \times \text{vol of buffer used (20 ml)} \times \frac{1}{\text{area of vial (6.25 cm}^2)}$$

where PH is the peak height.

The reason for obtaining units of μg/min$^{1/2}$ is to allow for a comparison of the release rate of indomethacin and the fluxes of indomethacin through snake skin. The release rates of indomethacin from the petrolatum ointments are linearly dependent on the concentration of indomethacin incorporated in the ointments for up to 1% (Fig. 5).

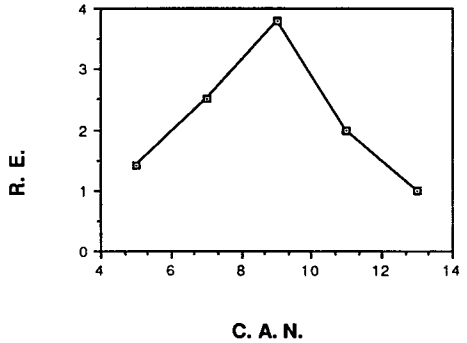


Fig. 3. The parabolic relationship between the relative enhancement and the carbon atom number of the alkyl chain of alkyl dimethyl amino acetates.

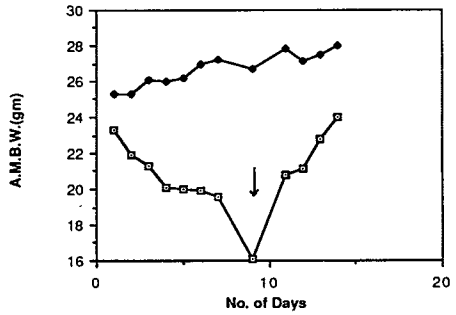


Fig. 4. A plot of the average mice body weight (g) versus the number of days. The arrow indicates the last day of injection of dodecyl dimethyl amino acetate. (■) Control: 6 Swiss white mice injected with 2% Tween 80 saline solution. (⊡) Seven Swiss white mice injected with 1 g/kg of dodecyl dimethyl amino acetate in 2% Tween 80 saline dispersion.

Table IV. Indomethacin Release Rates at 32°C for Petrolatum Ointments with Different Concentrations of Indomethacin and 5% of Dodecyl *N,N*-Dimethylamino Acetate

% indomethacin	Release rate (μg/min$^{1/2}$/cm^2)
0.1	0.33
0.25	0.84
0.5	1.84
1.0	3.15

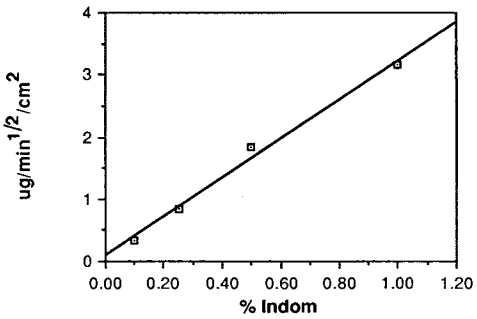


Fig. 5. A plot of the release rates of indomethacin versus the percentage of indomethacin incorporated in the petrolatum ointments.

Saturation Study

It is difficult to measure the saturation concentration of indomethacin in petrolatum using ordinary methods. A more practical way is to determine the penetration fluxes of indomethacin in petrolatum ointments with different indomethacin concentrations. If the fluxes depend on the concentration of solubilized indomethacin in petrolatum, then the fluxes will increase as the concentration of solubilized indomethacin increases until a saturation point is reached and the fluxes level off. This was observed in the present study. The steady-state fluxes obtained from the saturation study are summarized in Table V. They increase as the concentration of indomethacin increases but level off at 1% of indomethacin, which was assumed to be the approximate saturation concentration.

Concentration Effect of Dodecyl *N,N*-Dimethylamino Acetate on Penetration Enhancement

The steady-state fluxes, obtained from the time-course penetration profiles of indomethacin from ointments made of 1% of indomethacin and different concentrations of dodecyl *N,N*-dimethylamino acetate, are shown in Table VI. It is clear that the fluxes of indomethacin through snake skin increase linearly as the concentration of dodecyl *N,N*-dimethylamino acetate increases. A formulation consisting of 1% indomethacin, 5% dodecyl *N,N*-dimethylamino acetate, and 94% petrolatum produces a flux of indomethacin of 0.460 μg/hr/cm^2, which is equivalent to 0.06 μg/min$^{1/2}$/cm^2. The flux of 0.06 μg/min^2/cm^2 can be compared with the release rate of indomethacin (3.2 μg/min$^{1/2}$/cm^2) from the same formulation. By this comparison, the release rate of indomethacin is 53 times faster than its penetration rate, assuming that the release rate of indomethacin from the release study and the steady-state fluxes from the penetration study are both determined under a sink condition. Thus, the release and enhancer concentration effect studies indicate that the penetration of snake skin is the rate-limiting step.

Table V. Concentration Effects of Indomethacin on Its Penetration Flux at 32°C

% indomethacin	SS flux ± SD[a] (μg/cm^2 · hr)	t_L (hr)[b]
0.1	0.090 ± 0.016	5.0
0.25	0.18 ± 0.049	9.4
0.5	0.32 ± 0.12	6.1
1.0	0.66 ± 0.12	7.3
1.0[c]	0.74 ± 0.16	4.2
2.0[c]	0.84 ± 0.10	3.6
5.0[c]	0.84 ± 0.057	1.9
10.0[c]	0.76 ± 0.10	5.9

[a] SD = $\sqrt{[\Sigma (X_i - X)^2]/N-1}$ where X_i = individual value, X = mean value, and N = number of degrees of freedom.
[b] Calculated from the analytical expressions obtained from linear regression of the steady states of the penetration profiles.
[c] Snake skins were obtained from a different snake.

Table VI. Effect of the Concentration of Dodecyl *N,N*-Dimethylamino Acetate on the Flux of Indomethacin at 32°C

% of enhancer	Flux[a] ± SD[b] (μg/hr/cm^2)	Flux[c] (μg/min$^{1/2}$/cm^2)
2.5	0.31 ± 0.014	0.04
5.0	0.46 ± 0.24	0.06
7.5	0.76 ± 0.012	0.1
10	1.14 ± 0.24	0.15
15	1.59 ± 0.12	0.18

[a] Obtained directly from the linear plots of the steady state of the penetration profiles.
[b] SD = $\sqrt{[\Sigma (X_i - X)^2]/N-1}$ where X_i = individual value, X = mean value, and N = number of degrees of freedom.
[c] Obtained by dividing the fluxes in column 2 by the square root of 60 min.

Effect of Pretreatment of Snake Skin with Dodecyl *N,N*-Dimethyl Acetate

In order to see if the new enhancers acted directly on the skin, snake skin was pretreated with dodecyl *N,N*-dimethylamino acetate. If the enhancer interacts with the skin and increases skin permeability to indomethacin, one should see higher penetration fluxes in the pretreatment trials than in the controls. This was observed in Fig. 6. The penetration enhancement of indomethacin was indeed twofold, suggesting that the skin was the target of the enhancement effect.

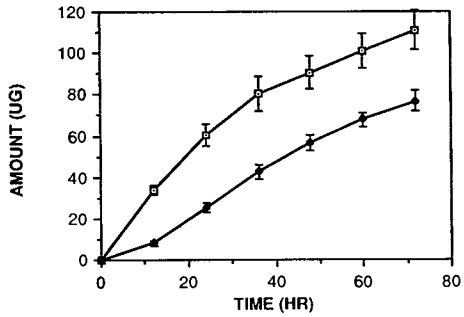


Fig. 6. Time-course penetration profiles of indomethacin through pretreated snake skin at 32°C for ointments with 5% dodecyl *N,N*-dimethylamino acetate. (□) Snake skins pretreated with dodecyl *N,N*-dimethylamino acetate. (◆) Control (skins not pretreated).

Electron Micrograph Study

To test whether the enhancers changed the skin structure, electron micrograph studies were carried out. The shed snake skin of *Elaphe obsoleta* has three different layers constituted mainly of keratin and lipids. The outermost layer is called the beta layer (beta-keratin rich), the middle layer is called the mesos layer (alpha-keratin and lipid rich), and the innermost layer is called the alpha layer (alpha-keratin rich) (15). The mesos layer has been suggested as being similar to the human stratum corneum (15). Figures 7a, b, and c show the scanning electron micrographs of shed snake skin for the control, the skin treated with Azone, and the skin treated with dodecyl *N,N*-dimethylamino acetate, respectively. There is no distinguishable change on the surface of the skin. Light transmission micrographs of skin cross section are shown in Figs. 8a, b, and c. Comparing the light transmission micrograph of shed snake skin with the control, Azone does not seem to change the beta, mesos, or alpha layers significantly. However, skin treated with dodecyl *N,N*-dimethylamino acetate had changed in two ways. First, the skin had swollen to 1.5 times of the control, in particular the beta layer. Second, the mesos layer was fused with its adjacent layers so that its clear layers are no longer seen between the outermost and the innermost layers.

Penetration of Indomethacin Through Snake Skin from Solution

The experimental data presented in this work show clearly that the new enhancers improve the indomethacin transport mainly by reducing the skin resistance. Since the formulations used in the above experiments were semisolid ointments, the transport of indomethacin from ointments to receptor cells is an overall process of at least two steps: release of indomethacin from ointment and penetration of the skin. A solution formulation would eliminate the drug release step. Table VII shows the data of a one-point sampling study. After 24 hr, there is only 2 μg of indomethacin detected for the solution formulation without enhancer. However, 21.7 μg of indomethacin (about 11 times of the control) was detected at the same hour for the solution formulation with 5% of dodecyl *N,N*-dimethylamino acetate. It is exciting to note that 364.5 μg of indomethacin was detected in the receptor cells at 24 hr for skins pretreated with the enhancer. This means that the skin resistance has been reduced remarkably. Further, at pH 7.2, where indomethacin (pK_a 4.5) (17) is in mainly the ionized form, its penetration flux is high in the presence of the enhancer. This issue has been investigated further by studying the penetration of the nonionized and ionized species of a basic drug (clonidine) and an acidic drug (indomethacin) in the presence and absence of dodecyl *N,N*-dimethylamino acetate using snake skin as the skin model (18). The results of this study indicate that the permeability coefficient of the nonionized species of indomethacin (3.62×10^{-3} cm/hr) is much higher than that (2.19×10^{-5} cm/hr) of the ionized species in the absence of the enhancer. In the presence of the enhancer the permeability coefficient of the ionized species of indomethacin has been increased by a factor of 36, although that of the unionized species remains unaltered. Therefore, the total penetration fluxes of indomethacin were controlled primarily by the concentrations of the species in the donor cell and their permeability coefficients. These penetration characteristics are consistent with the high penetration flux of indomethacin at pH 7.2 observed in the present study.

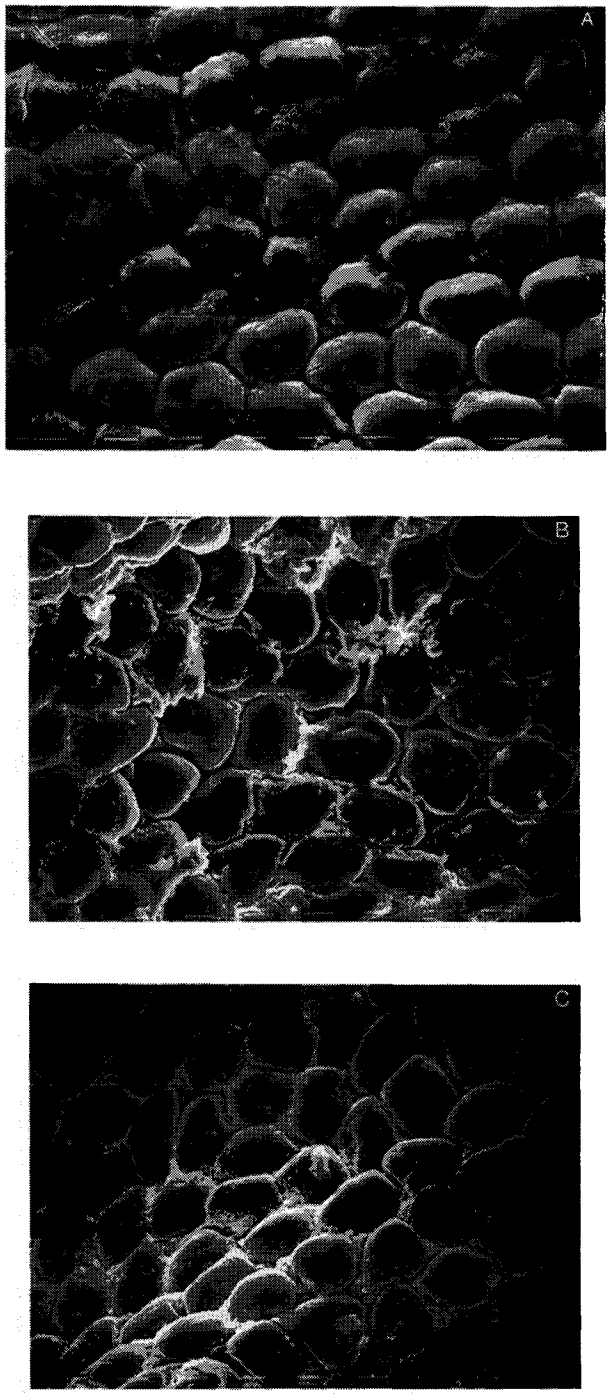


Fig. 7. Scanning electron photomicrograph of the surface of shed snake skin (*Elaphe obsoleta*). (a) Control, ×640, reduced 45% for reproduction; (b) skin treated with Azone, ×640, reduced 35%; (c) skin treated with dodecyl *N,N*-dimethylamino acetate. ×640, reduced 35%.

Irritation Potential

The preliminary irritation study conducted by applying

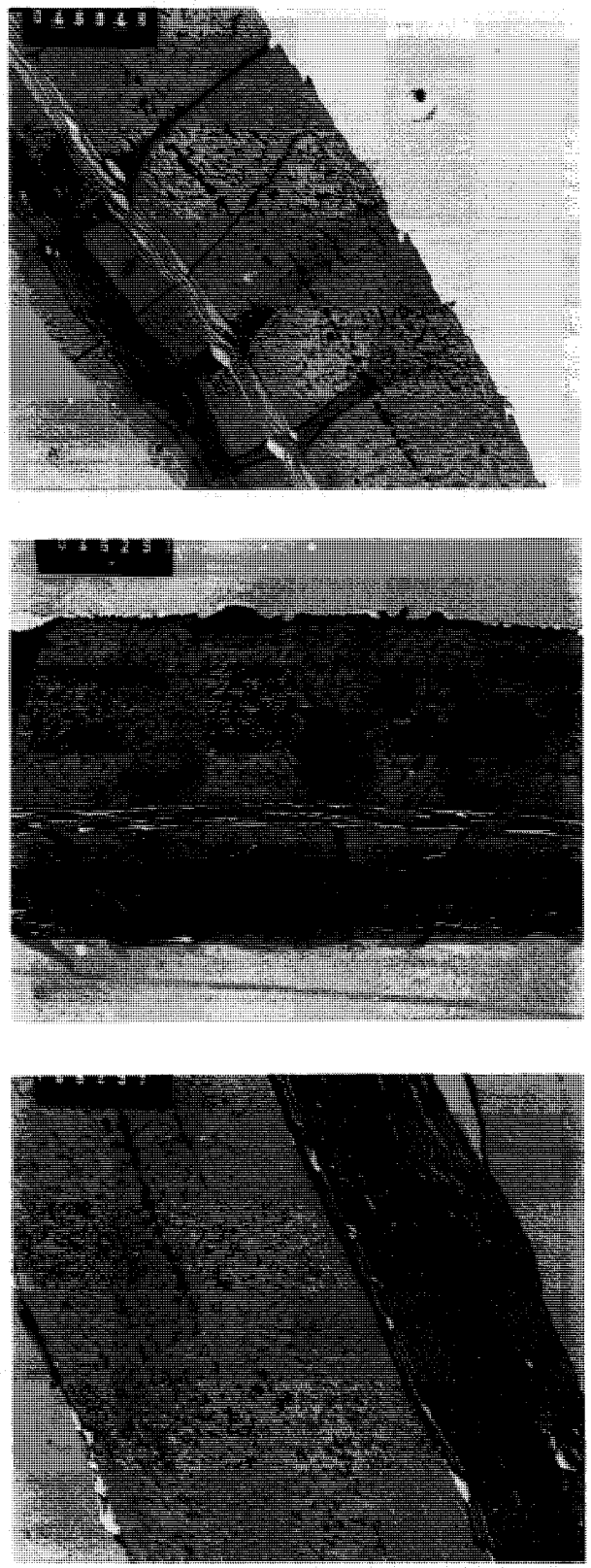


Fig. 8. Light transmission photomicrograph of cross section of shed snake skin (*Elaphe obsoleta*). (a) Control; (b) skin treated with Azone; (c) skin treated with dodecyl *N,N*-dimethylamino acetate. ×3300; figs. reduced 40% for reproduction.

Table VII. Penetration of Indomethacin Through Shed Snake Skin at 32°C from Solution Formulations Using Dodecyl *N,N*-Dimethylamino Acetate as the Penetration Enhancer

Formulation	Amount of indomethacin (μg)[a]
i[b]	1.93 ± 0.01
ii[c]	28 ± 10
i[d]	364 ± 2

[a] Detected in the receptor cells after 24 hr.
[b] Control; 1% indomethacin (w/v), 2% Tween 80 (w/v), and pH 7.2 phosphate buffer (3.92 ml).
[c] 1% indomethacin (w/v), 2% Tween 80, 5% dodecyl *N,N*-dimethylamino acetate, and pH 7.2 phosphate buffer (1.84 ml).
[d] Snake skins pretreated with dodecyl *N,N*-dimethylamino acetate.

nine doses of 1% indomethacin petrolatum ointment consisting of 5% of dodecyl *N,N*-dimethylamino acetate on the same area of the hairless mouse shows no irritation on the hairless mouse skin as evidenced by the absence of redness on the skin. It has been suggested that hairless mouse skin is more sensitive than human skin, and it is often used for chemical irritation testing (19).

Conclusions

A series of alkyl *N,N*-disubstituted amino acetates with a low toxicity and excellent penetration enhancing effects on indomethacin has been prepared. This type of new enhancer may be nonspecific in transdermal drug delivery, with broad applications for different drugs.

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