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Transdermal penetration enhancers in rabbit pinna skin: Duration of action, skin irritation, and in vivo/in vitro comparison

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Summary

Irritation of the skin by chemical penetration enhancers may limit the use of these compounds in transdermal drug delivery. Biodegradable enhancers like dodecyl *N,N*-dimethylamino acetate (DDAA) have been synthesized previously to decrease the duration of action and toxicity of the enhancers. We studied the reversibility and extent of penetration enhancement and skin irritation by DDAA, Azone, and *n*-dodecanol in rabbit pinna skin using timolol and propranolol as penetrants. Also, in vitro and in vivo permeabilities of the drugs with and without enhancers were compared. Drug concentrations in diffusion chambers and rabbit plasma were determined using HPLC and radio receptor assay, respectively. Skin irritation was measured with a chromameter. DDAA and Azone caused approximately equal transdermal penetration enhancement of model drugs in vitro but the potency of *n*-dodecanol was lower. In vivo, Azone was the most irritating enhancer in rabbit pinna skin. Both enhancer effects and skin irritation by DDAA were reversed in 4 days, while the effects of Azone and *n*-dodecanol lasted longer. Thus, it is possible to affect the duration of skin alteration by enhancer design. Propranolol was more irritating than timolol in rabbit pinna skin in vivo. Percutaneous permeability of propranolol in vivo, calculated from pharmacokinetic parameters, was considerably greater than its in vitro permeability coefficient. In contrast, in vitro and in vivo permeability coefficients of timolol were comparable. The increased permeation of propranolol in vivo may be due to skin irritation, because in vivo permeability coefficients correlated with associated skin irritation.

Introduction

Due to its possibilities as an alternative route of drug administration, transdermal drug administration is currently being studied widely (Chien, 1991). Poor permeability in stratum corneum limits the usefulness of the transdermal drug administration route. Drug penetration through the stratum corneum can be increased with penetration enhancers, compounds that make the skin more permeable to drugs (Barry, 1987). Penetration enhancers are, however, potential skin irritants and their systemic toxicity is a concern. The relationship between skin irritation and transdermal penetration enhancement is unclear.

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In order to decrease local and systemic toxicity, biodegradable penetration enhancers that have reversible action in the skin and safe degradation products have been synthesized (Wong et al., 1989). One of these enhancers, dodecyl *N,N*-dimethylamino acetate (DDAA), is degraded by esterase catalyzed hydrolysis to *N,N*-dimethylglycine and *n*-dodecanol (Buyuktimkin et al., 1991). Since esterases are also present in the epidermis and dermis of the skin, local biodegradation of DDAA is probable (Martin et al., 1987). DDAA increases the permeability of many drugs in human, shed snake, and rabbit pinna (ear) skin in vitro in diffusion cells (Wong et al., 1989; Hirvonen et al., 1991). However, the duration and extent of penetration enhancement and irritation by DDAA have not been studied in vivo.

Although the permeabilities of many drugs in rabbit skin exceed that of human skin (Hirvonen et al., 1991), the rabbit is a convenient model in transdermal in vivo absorption studies and it is also sensitive to skin irritation. In this study, the effects of DDAA on skin irritation and transdermal drug penetration were compared to Azone and *n*-dodecanol. Azone was chosen as a well known non-degradable enhancer and *n*-dodecanol as the degradation product of DDAA.

Materials and Methods

Animals

New Zealand White rabbits (2.2–3.0 kg) of both sexes were used. Before the in vivo tests, the rabbits were housed singly in cages under standard laboratory conditions (10 h dark/ 14 h light/ day; temperature 20°C; relative humidity 65%). The animals were fed with standard laboratory pellets and allowed to drink tap water ad libitum. During the tests rabbits were kept in boxes where they could move their heads freely. The total number of the rabbits in these studies was 45.

Chemicals

Dodecyl *N,N*-dimethylamino acetate (DDAA) was kindly provided by Dr J. Howard Rytting (Dept of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS). Azone (Whitby Research Inc., Irvine, CA) and *n*-dodecanol (Sigma Chemical Co., St Louis, MO) were purchased. The model drugs were propranolol HCl (AMSA, Milano, Italy) and timolol maleate (Leiras, Tampere, Finland). Hydroxypropyl methylcellulose (HPMC) had a molecular weight of 80000 and was purchased from Aldrich-Chemie (Steinheim, Germany). The animals were killed by T-61 Euthanasia solution (Hoechst, Munich, Germany). Tritiated water was from Du Pont, New England Nuclear Products (Boston, MA; specific activity 1 mCi/g). Other chemicals used were of standard laboratory quality. [^{3}H]L-CGP12177 had a specific activity of 45 Ci/mmol, radiochemical purity of 96% and was from Amersham International (Buckinghamshire, U.K.).

In vitro permeation

In in vitro studies, rabbit pinna (ear) skin was separated from the middle of the inner side of the rabbit ear (Corbo et al., 1990). The pinna skin was peeled away from the underlying cartilage, immediately after the animals were killed. The effect of DDAA, Azone, and *n*-dodecanol on the permeation of propranolol and timolol across the rabbit pinna skin in vitro was studied in modified Franz diffusion cells (Crown Glass Co. Inc., Somerville, NJ) by pretreating the skin samples with 10 μl of pure DDAA, Azone or *n*-dodecanol 3.5 h before experiments. Thereafter, 1.0 g of propranolol (100 mg/ml) or timolol (75 mg/ml) in 5% HPMC at pH 7.0 was placed into the closed donor chamber of the diffusion cell on the skin for 72 h. The receiver phase (5.0 ml) was pH 7.4 phosphate buffer, the area of exposed skin was 0.64 cm^2, and the temperature was 37°C. Samples of 250 μl were withdrawn from the receiver compartment at fixed times and replaced with blank buffer solution. Four to six parallel experiments were conducted in each case.

Propranolol and timolol were analyzed by RP-HPLC (Beckman System Gold, Beckman Instruments Inc., San Ramon, CA), using a Supelco LC-18-DB Column (5 μm, 150 × 4.6 mm; Supelco Inc., Rohm and Haab Co., Bellefonte, PA). The mobile phase was 35% acetonitrile/65% acetic acid at pH 4.0 for propranolol and 30% ACN/70% acetic acid at pH 4.0 for timolol. The

wavelengths of UV detection for propranolol and timolol were 289 and 294 nm, respectively.

In vitro steady-state drug fluxes, J_{ss} (μg h^{-1} cm^{-2}) were calculated using the linear portion of drug penetrated vs time plots employing least squares linear regression. In vitro permeability coefficients ($P_{\text{in vitro}}$) of propranolol and timolol in rabbit skin were calculated as $P_{\text{in vitro}} = J_{ss}/C_d S$, where C_d is the drug concentration in the donor solution and S denotes the surface area of the skin.

In vivo absorption

To measure transdermal permeability in vivo in the control situation, propranolol (100 mg/ml) or timolol (75 mg/ml) in HPMC were applied to the center of rabbit pinna skin for 4 h in two circular plastic containers. The exposed skin area was 3.5 cm^2. Only one ear of each rabbit was used.

The extent and duration of increased permeability and irritation of the skin were studied after application of 55 μl of pure liquid DDAA, Azone, or *n*-dodecanol. At different times after enhancer, timolol and propranolol were applied on the rabbit pinna skin in HPMC as in the control case. Due to severe skin irritation, only the effects of DDAA and Azone on propranolol absorption were studied. In the case of timolol all three enhancers were tested in vivo. Three to five parallel experiments were carried out for each enhancer pretreatment and control.

After timolol or propranolol administration blood samples of 1.5 ml were taken from the cannulated contralateral ear artery at fixed times up to 4 h. The concentration of propranolol or timolol in plasma was determined using the radioreceptor assay of Wellstein et al. (1984), as modified by Urtti and Kyyrönen (1989). In the assay, a plasma sample of timolol or propranolol plasma displaces the radioligand, [^{3}H]L-CGP12177 from β_2-receptors of isolated rat reticulocytes. After incubation receptor-bound and free radioligand were separated using vacuum filtration as described earlier (Wellstein et al., 1984). Radioactivity of the filters was analyzed by liquid scintillation counting as described by Urtti and Kyyrönen (1989). Non-specific binding of the radioligand to the membranes ($< 5\%$ of the total binding) was determined by incubating the radioligand, reticulocytes and blank plasma in 2.5×10^{-5} M propranolol or timolol. Receptor-bound radioactivity was calculated by subtracting the non-specific binding from the total binding of radioligand. The samples were analyzed in triplicates and the mean values were used in the calculations. Five standard concentrations of model drugs were incubated with each run. Standard curves were linear from 5 to 40 nM of propranolol and from 0.5 to 20 nM of timolol in the incubation vials. The results were calculated as ng/ml of model drug equivalents in plasma. The method measures propranolol, timolol and their possible active metabolites.

Determination of pharmacokinetic parameters

For the in vivo calculations, four rabbits received an i.v. bolus of propranolol (0.50 mg/kg) in normal saline into the marginal vein. Blood samples were collected from the contralateral cannulated ear artery for 120 min. Plasma was separated and propranolol concentration determined as described before. Pharmacokinetic parameters for propranolol in the rabbit were calculated using a two-compartment model and biexponential curve fitting (Eqn 2) (Gibaldi and Perrier, 1982):

$$c = Ae^{-\lambda_1 t} + Be^{-\lambda_z t} \tag{2}$$

AUC was calculated from

$$\text{AUC}_{0-\infty} = (A/\lambda_1) + (B/\lambda_z) \tag{3}$$

The clearances (CL) of propranolol and timolol in rabbit plasma were calculated as (Eqn 4):

$$\text{CL} = \text{D}/\text{AUC}_{0-\infty} \tag{4}$$

where D is the dose.

The steady-state drug concentration in vivo in rabbit plasma was determined as described earlier (Eller et al., 1985). A steady-state concentration was considered to be achieved when the analysis of variance indicated no significant difference ($P > 0.05$) between drug concentrations

of consecutive sampling times. From the concentrations at steady state, the mean value was calculated and used in further data analysis. Percutaneous flux of drug in vivo, J_{ss}, was calculated as:

$$J_{ss} = c_{ss} \times CL \quad (5)$$

where c_{ss} is the steady-state drug concentration. The percutaneous permeability coefficients of propranolol and timolol in vivo ($P_{in\ vivo}$) were calculated using Eqn 6:

$$P_{in\ vivo} = J_{ss}/C_d S \quad (6)$$

C_d denotes the drug concentration in the hydrogel formulation and S is the contact surface area.

Tritiated water flux

To determine possible changes in the skin after propranolol or timolol permeation in vivo, we measured in vitro the flux of tritiated water across the same skin which had previously been used in the in vivo drug permeation study. The skin samples were separated from the cartilage and placed into the diffusion cells as described above. Tritiated water was added to the donor phase (1.0 μg ^3H_2O in 1.0 ml pH 7.4 buffer). Samples were withdrawn from the receiver chamber and tritium was measured using liquid scintillation counting. The flux of ^3H_2O was determined similarly to that of the model drugs in the in vitro permeability study. The flux of ^3H_2O in separated rabbit pinna skin samples from in vivo experiments was compared to water flux across untreated rabbit skin.

Skin irritation

The skin irritation induced by the drugs, enhancers, and the combination of both was studied with a Minolta® Chromameter CR 200 (Minolta, Osaka, Japan). Color reflectance was recorded in a three-dimensional space, $L^*a^*b^*$, following the recommendations of the Commission Internationale d'Eclaire CIE (Wyszecki and Stiles, 1982). The L^* value (luminance) gives the relative brightness of the color, ranging from completely black ($L^* = 0$) to white ($L^* = 100$). The a^* value represents the balance between red and green ($+100$ to -100) and the b^* value expresses the yellow-blue axis ($+100$ to -100). On each day, the instrument was calibrated using a standard white plate.

Measurements of rabbit pinna skin irritation were performed by placing the colorimeter vertically onto the test site. The average of three measurements on each treatment was regarded as the skin irritation value (E):

$$E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

The E value corresponds to the change from the colorimetric values of the untreated skin before the test due to the treatment.

Statistics

The statistical significance of the differences between different treatments was analysed using Mann-Whitney's U-test.

Results

The permeability coefficients of timolol and propranolol in the rabbit pinna skin in vitro were 4.6 and 5.8×10^{-8} cm/s, respectively. All en-

TABLE 1

Permeability of timolol and propranolol from hydrogel in rabbit pinna skin in vitro

Test	Permeability ± SE (10^{-8} cm/s)	Enhancement factor [a]
Timolol		
Control	4.6 ± 1.1	–
DDAA [b]	284 ± 17.1	61.9
Azone	271 ± 18.9	59.0
Dodecanol	168 ± 9.9	36.6
Propranolol		
Control	5.8 ± 0.9	–
DDAA	223 ± 10.4	38.4
Azone	159 ± 6.9	27.4
Dodecanol	153 ± 15.8	26.3

Penetration enhancers were applied 3.5 h before the drugs ($n = 4$–6).

[a] Permeability with enhancer divided by drug permeability without enhancer.

[b] Dodecyl *N,N*-dimethylamino acetate.

hancers increased the permeability of timolol and propranolol in vitro considerably (Table 1). Penetration enhancement of timolol and propranolol ranged from approx. 37- to 62-fold and 26- to 38-fold, respectively. In the case of timolol DDAA and Azone caused equal penetration enhancement, while *n*-dodecanol was less effective. For propranolol DDAA was a more effective penetration enhancer than Azone or *n*-dodecanol (Table 1).

After i.v. injection of propranolol the best curve fit to the drug concentrations in plasma vs time was obtained with the equation: $c = 94.3$ ng/ml $e^{(-40.8\ h^{-1}\ t)} + 32.2$ ng/ml $e^{(-2.9\ h^{-1}\ t)}$. $AUC_{0-\infty}$ of intravenous propranolol was 5.9 h ng ml^{-1} kg^{-1} at a dose of 0.50 mg/kg and total plasma clearance 35.5 l h^{-1} kg^{-1}.

For timolol, the previously determined (Finne and Urtti, 1992; Järvinen et al., 1992) value of $AUC_{0-\infty}$ of 2.8 h ng ml^{-1} kg^{-1} at a dose of 35 μg/kg was used in the calculations. Clearance of timolol from rabbit plasma was 3.6 l h^{-1} kg^{-1}.

Steady-state concentrations of propranolol and timolol with and without penetration enhancers were achieved in rabbit plasma at 90–120 and 180–210 min, respectively (Fig. 1). In the case of timolol, penetration enhancer pretreatment 3.5 h before drug application increased the permeability coefficient of timolol 43–117-fold compared to skin without enhancer pretreatment (Table 2).

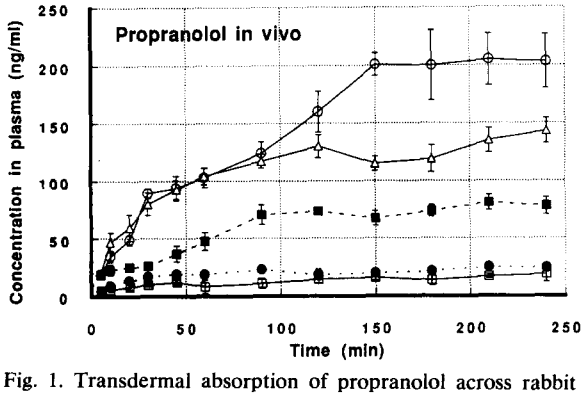

Fig. 1. Transdermal absorption of propranolol across rabbit pinna skin in vivo. Propranolol concentrations in rabbit plasma after DDAA pretreatment 3.5 (○), 24 (△), 48 (■), and 96 h (●) before drug and without enhancers (□) are presented. Means ± SE ($n = 3$–5).

TABLE 2

Permeability of timolol and propranolol in rabbit pinna skin in vivo

Test	Permeability ± SE (10^{-8} cm/s)	Enhancement factor [a]
Timolol		
Control	5.7 ± 0.8	–
DDAA 3.5 h	298 ± 54	52
Azone 3.5 h	668 ± 42	117
N-Dodecanol 3.5 h	247 ± 31	43
DDAA 96 h	10.2 ± 1.0	1.8
Azone 96 h	182 ± 22	32
N-Dodecanol 96 h	102 ± 13	18
Propranolol		
Control	82 ± 16	–
DDAA 3.5 h	1140 ± 71	14
Azone 3.5 h	1113 ± 59	14
DDAA 24 h	715 ± 41	8.7
DDAA 48 h	419 ± 22	5.1
DDAA 96 h	118 ± 12	1.4
Azone 96 h	761 ± 60	9.2
Azone 240 h	111 ± 12	1.3

Penetration enhancers were applied at different times before the drug ($n = 3$–5).

[a] Permeability with enhancer divided by drug permeability without enhancer.

Azone was the most effective promoter of timolol permeation. In the case of propranolol, the magnitude of penetration enhancement by Azone and DDAA was 14-fold. However, the permeability coefficients of propranolol were higher than those of timolol.

At 96 h after DDAA pretreatment, the permeability coefficient of propranolol and timolol in rabbit skin had returned close to the level of untreated skin (Table 2). In the case of Azone or *n*-dodecanol pretreatment, the enhancement was still about one order of magnitude at 96 h. At 240 h after Azone pretreatment, the permeability of propranolol was not greater than that of the control ($P > 0.05$).

The permeability coefficient of propranolol in all cases ($P < 0.05$) and the permeability of timolol after Azone pretreatment were significantly higher in vivo than in vitro ($P < 0.05$). In other cases, timolol had similar permeability in vivo and in vitro (Tables 1–3).

TABLE 3

In vivo / in vitro ratios of timolol and propranolol permeability coefficients in rabbit pinna skin

Test	$P_{\text{in vivo}} / P_{\text{in vitro}}$	
	Timolol	Propranolol
No enhancers	1.2	14.2
DDAA	1.1	5.1
Azone	2.5	7.0
Dodecanol	1.5	– [a]

Enhancers were applied 3.5 h before drug in vitro and in vivo.
[a] Not done.

The in vitro fluxes of tritiated water in the skin samples were 1.13 and 1.03% h^{-1} cm^{-2}, respectively. Tritium fluxes in the skin samples, which had been used in in vivo propranolol or timolol permeation experiments without enhancers, were 2.86 and 1.20% h^{-1} cm^{-2}, respectively.

Skin irritation decreased the brightness of the skin (L^*) and increased redness of the skin (a^*), but the change in b^* values (blue/yellow) was negligible. This was confirmed with visual observations. The higher E values also corresponded to the greater skin irritation (redness) visually. In the case of Azone, the skin irritation was more intense than after DDAA or *n*-dodecanol application and the irritation increased with time. After application of DDAA and *n*-dodecanol, the skin irritation decreased with time (Fig. 2). Propranolol was more irritating than timolol in rabbit pinna skin, and the skin irritation induced by propranolol increased after the gel formulation was removed from the skin (Fig. 3). Timolol induced only a mild reaction, which decreased with time.

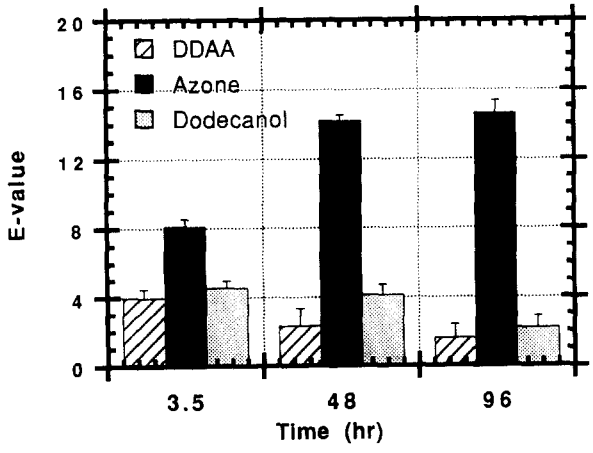

Fig. 2. Development of skin irritation (chromametric E values) in rabbit pinna skin after single application of 55 μl of pure liquid enhancers. Control value = 0. Mean ± SE, $n = 3$.

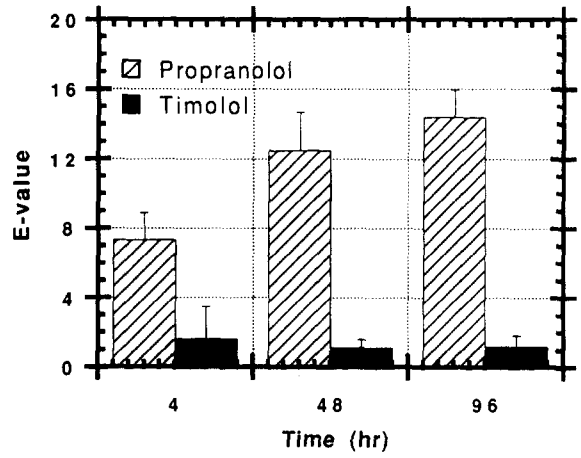

Fig. 3. Development of skin irritation in rabbit pinna skin after 4 h of timolol or propranolol exposure in hydrogel formulation. Mean ± SE, $n = 3$.

The skin irritation at 4 h after propranolol exposure is shown in Fig. 4. When the skin was pretreated with DDAA 3.5 h before drug applica-

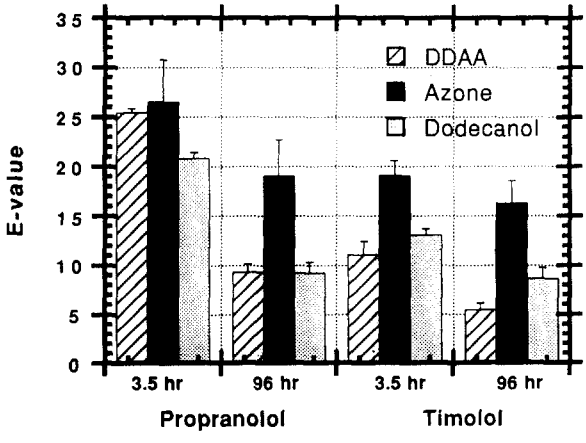

Fig. 4. Skin irritation (chromametric E values) in rabbit pinna skin 4 h after timolol or propranolol application in HPMC. The rabbit ears were pretreated with penetration enhancers 3.5 or 96 h before drug application. Means ± SE ($n = 3$) are presented.

tion severe irritation resulted (Fig. 4). When pretreatment was carried out 96 h before propranolol application, the resulting irritation was much milder in DDAA and *n*-dodecanol groups than in the case of Azone (Fig. 4). The irritation caused by the timolol hydrogel after enhancer pretreatment was lower than in the case of propranolol, but again Azone pretreatment led to more intense irritation than DDAA or dodecanol.

Discussion

Skin irritation by drugs and enhancers is a commonly encountered problem in transdermal drug delivery, and limits the use of the transdermal route in drug delivery (Lammintausta et al., 1988; Finne and Urtti, 1992). For example, the transdermal route is a promising alternative for the delivery of timolol (Cargill et al., 1986; Kubota et al., 1991). Propranolol has been used as a model drug in transdermal rabbit studies in vivo (Corbo et al., 1989, 1990), however, in this study propranolol induced skin irritation in rabbits.

The 14-fold increase in permeability coefficient of propranolol in vivo compared to the in vitro situation (Table 3) was probably due to skin irritation (Fig. 3). The permeability coefficient of timolol in vivo was similar to its permeability in vitro (Table 3) and timolol did not induce skin irritation. Increased skin permeability induced by propranolol in vivo is further supported by the 2.6-fold increase in water permeability in the treated skin samples. Again, this kind of increase was not observed in timolol-treated skins. Neither propranolol nor timolol affected the permeation of tritiated water across the rabbit pinna skin in vitro, which suggests that the drugs do not cause direct chemical damage to the stratum corneum (Hirvonen et al., 1991). Thus, the skin irritation by propranolol may increase the permeability of the skin through an indirect mechanism involving a physiological skin response. Increased permeability then allows greater penetration of propranolol which may further increase the irritation as seen in Fig. 2.

Drug permeability as a function of skin irritation (with and without penetration enhancers) is

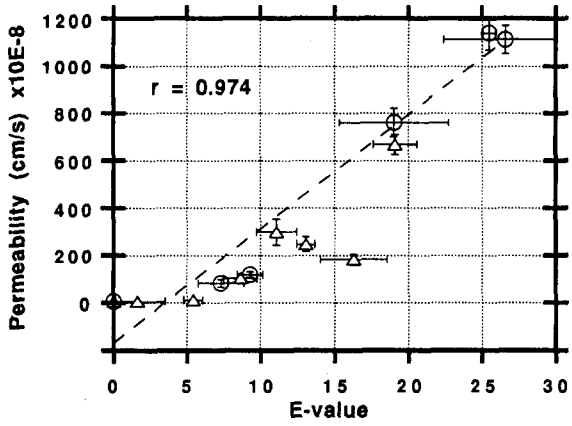

Fig. 5. In vivo permeability coefficient vs skin irritation plot of propranolol (circles) and timolol (triangles) in rabbit pinna skin. The data with and without penetration enhancers (DDAA, Azone, and *n*-dodecanol) have been pooled. Irritation (E value) of zero represents in vitro situation. Mean ± SE, $n = 3$–6.

presented in Fig. 5. The linear correlation coefficient of 0.974 suggests that in vivo increased drug permeability is related to skin irritation. It should be emphasized that the in vivo and in vitro permeation of drugs may not necessarily be similar even without associated irritation. In this study, however, the permeability coefficient of the least irritating test system (timolol without enhancers) was similar in vitro and in vivo (Table 3).

DDAA, Azone and *n*-dodecanol are lipophilic permeation promoters, and their main mechanism of action is the disordering of stratum corneum lipid domains (Barry, 1987; Hirvonen et al., unpublished results). Therefore, a strong permeation enhancing effect with the relatively lipophilic model drugs, propranolol and timolol, in vitro and in vivo was expected. Although DDAA seemed to be the most efficient enhancer, *n*-dodecanol, the degradation product of DDAA, also increased the permeation of propranolol and timolol substantially both in vitro and in vivo (Tables 1 and 2). Interestingly, the enhancing effect of DDAA on skin permeation of propranolol and timolol was almost completely reversed in 4 days, while Azone and dodecanol still showed a substantial effect. There are several possible reasons for the shorter duration of action of DDAA compared to Azone and dodecanol.

Firstly, due to their higher lipophilicities, dodecanol and azone may be retained for a longer period in the stratum corneum than DDAA. Because of its hydrophilic end, DDAA is less lipophilic than Azone (log $P = 6.60$ (Barry, 1987)) and *n*-dodecanol (log $P = 5.13$ (Leo et al., 1971)). For example, Wiechers et al. (1987) were able to detect Azone in human stratum corneum at least 3 days after application on the skin.

Secondly, hydrolysis of DDAA by the esterases to dodecanol and *N,N*-dimethylglycine in the viable epidermis and dermis may help to maintain a steep concentration gradient of DDAA between the stratum corneum and viable tissue. Part of the formed dodecanol may diffuse back to the stratum corneum, but apparently this is not significant, as demonstrated by the more rapid reversibility of skin after DDAA than dodecanol treatment (Table 2).

Thirdly, the efficient and long-lasting drug penetration enhancement after Azone pretreatment can partly be explained by the skin irritation. Irritation increases penetration and vice versa. This interplay prolongs the skin irritation. This factor, however, does not explain the different durations of activity between DDAA and dodecanol as these enhancers caused similar irritation in the skin.

Scaling of these results to humans is difficult, since rabbit skin is more permeable than human skin (Hirvonen et al., 1991). Also, in human volunteer skin, the irritation induced by Azone is relatively low suggesting lower irritability of human skin (Wiechers et al., 1987). Nevertheless, it is possible that irritation could also cause increased drug permeabilities in human skin.

In conclusion, skin irritation induced by DDAA was considerably lower than that in the case of Azone. Biodegradation and faster elimination into the systemic circulation are favourable properties in decreasing the skin irritation.

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