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Research Paper

Synthesis and Enhancing Effect of Transkarbam 12 on the Transdermal Delivery of Theophylline, Clotrimazole, Flobufen, and Griseofulvin

Alexandr Hrabálek,[1,2,4] Pavel Doležal,[3] Kateřina Vávrová,[1,2] Jarmila Zbytovská,[3] Tomáš Holas,[1] Jana Klimentová,[1] and Jakub Novotný[1]

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Purpose. Dodecyl-6-aminohexanoate (DDEAC) is a transdermal permeation enhancer with excellent activity, low toxicity, and no dermal irritation. We hypothesized that DDEAC reacts with air CO_2 to form a two-chain ammonium carbamate—Transkarbam 12 (T12)—which is responsible for the enhancing effect.

Methods. DDEAC and T12 were synthesized, their structures were confirmed by spectral methods, and their enhancing activity was studied using the Franz diffusion cell and human skin. A high-performance liquid chromatography method was developed for determination of T12, and its biodegradability was evaluated using porcine esterase.

Results. Only the carbamate salt T12 was responsible for the high enhancing activity; DDEAC tested under argon to avoid reaction with CO_2 was inactive. T12 enhanced transdermal permeation of drugs covering a wide range of physicochemical properties, including theophylline (enhancement ratio up to 55.6), clotrimazole (7.7), flobufen (5.0), and griseofulvin (24). The activity was pH-dependent, further confirming the importance of the carbamate structure. The metabolization of T12 followed a second-order kinetics with $t_{1/2}$ = 31 min.

Conclusion. Our results indicate that T12 is a promising biodegradable permeation enhancer for a wide range of drugs, and the structurally novel group of carbamate enhancers warrants further investigation.

KEY WORDS: ammonium carbamate; biodegradability; permeation enhancer; transdermal drug delivery.

INTRODUCTION

Transdermal drug delivery offers numerous advantages over the conventional routes of administration; however, poor permeation of most drugs across the skin barrier constitutes a serious limitation of this methodology. One of the approaches used to enlarge the number of transdermally applicable drugs uses permeation enhancers. These compounds promote drug permeation through the skin by a reversible decrease of the barrier resistance (1).

1-Dodecylazepan-2-one (Azone) was the first molecule, specifically designed as a skin permeation enhancer (Fig. 1) (2). This compound enhances the skin transport of a wide variety of drugs (for reviews on Azone, see (3,4)) and serves as a lead compound for structural modifications (5–9). The structure–activity relationships of amphiphilic permeation enhancers have been reviewed recently (10).

We have previously reported the permeation-enhancing activity of a series of 6-aminohexanoic acid esters (11,12). The compounds were designed as acyclic Azone analogs (Fig. 1), the flexible structure of which could adopt an optimum conformation, and thus interact more readily with the stratum corneum components. Moreover, the ester linkage offers the possibility of degradation by skin esterases into nontoxic metabolites. These compounds displayed excellent enhancement activities for theophylline, approximately an order of magnitude higher than that of Azone—enhancement ratios of dodecyl-6-aminohexanoate (DDEAC) and Azone dispersed in water were 35.0 and 5.4, respectively, and 16.7 and 1.1, respectively, when applied in olive oil. The acute toxicity of DDEAC after intraperitoneal administration to mice was lower than that of Azone [LD_{50} of DDEAC and Azone were 352 mg/kg (11) and 232 mg/kg (13), respectively], and the compound did not exhibit any acute dermal irritation *in vivo* on rabbits (11).

Recently, we have found that the behavior of the enhancer does not correspond to that of an amino ester molecule, with the sample, moreover, containing only 91% of DDEAC. This finding could be explained by the formation

[1] Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 50012 Hradec Králové, Czech Republic.
[2] Centre for New Antivirals and Antineoplastics, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 50012 Hradec Králové, Czech Republic.
[3] Department of Pharmaceutical Technology, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 50012 Hradec Králové, Czech Republic.
[4] To whom correspondece should be addressed. (e-mail: Alexandr.Hrabalek@faf.cuni.cz)

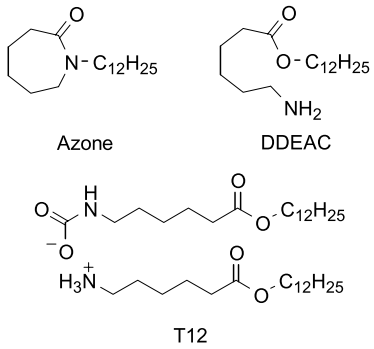

Fig. 1. Azone, its acyclic analog dodecyl-6-aminohexanoate (DDEAC), and the corresponding alkylammonium–alkylcarbamate Transkarbam 12 (T12).

of an ammonium carbamate salt by the trapping of carbon dioxide by the free amino group. This two-chain enhancer with a carbamate salt forming its polar head, termed Transkarbam 12 (T12, Fig. 1), has been suggested to be responsible for the enhancing effect (14,15).

The purpose of this study was to confirm the structure of this promising enhancer and evaluate its activity in a greater detail. We aimed at preparing both possible structures, DDEAC and T12, in pure form, and directly comparing their enhancing activities to find the contribution of both structures to the permeation-enhancing effect. In addition, we have evaluated the enhancing activity of T12 toward a broader spectrum of drugs, a preliminary *in vitro* biodegradability using porcine esterase, and developed a high-performance liquid chromatography (HPLC) method for the determination of this substance.

MATERIALS AND METHODS

Chemicals and Instrumentation

All chemicals, HPLC solvents, theophylline, clotrimazole, griseofulvin, and esterase [from porcine liver, suspension in 3.2 M $(NH_4)_2SO_4$, pH 8, 250 U/mg protein, 15 mg protein/ml] were purchased from Sigma-Aldrich (Schnelldorf, Germany). Flobufen (16) was obtained from VUFB (Prague, Czech Rep.). HPLC columns (see below) were purchased from Merck (Darmstadt, Germany).

Infrared (IR) spectra were recorded on a Nicolet Impact 400 apparatus equipped with a DTGS detector with a resolution of 4 cm^{-1}. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for ^1H and 75 MHz for ^{13}C. Fast atom bombardment (FAB) mass spectra were run on a ZAB-2SEQ mass spectrometer using a cesium ion gun at 25 kV. The sample was dissolved in toluene, and 1 μl of the solution was added to the matrix. For +FAB, 2-nitrophenyl(octyl)ether was used as a matrix and diethanolamine for −FAB. Elemental analysis (C, H, N) was performed on a Fisons EA 1110 CHNS-O elemental analyzer. The melting point was measured on a Kofler apparatus and is uncorrected.

Synthesis

Dodecyl-6-aminohexanoate. DDEAC was synthesized as described previously (11). To obtain pure amino ester without traces of a carbamate, the substance was heated at reflux in $CHCl_3$ for 5 min and was then dried using a stream of argon. The process was repeated twice, and the product was stored under argon to avoid interaction with carbon dioxide. MW = 299.5 g/mol. Colorless oil. Yield: 89%. IR ($CHCl_3$): ν_{max} 3448, 3375, 2928, 2856, 1725, 1467 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.04 (2H; t; J = 6.6 Hz, OCH_2); 2.69 (2H; t; J = 6.9 Hz, NCH_2); 2.29 (2H; t; J = 7.5 Hz, $COCH_2$); 1.86 (2H; bs, NH_2); 1.70–1.55 (4H; m; $2CH_2$); 1.55–1.40 (2H; m; CH_2); 1.40–1.15 (20H; m; $10CH_2$); 0.87 (3H; t; J = 6.9 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.80; 64.46; 41.82; 34.25; 33.04; 31.88; 29.60; 29.55; 29.49; 29.32; 29.22; 28.60; 26.37; 25.89; 24.76; 22.66; 14.10.

5-(Dodecyloxycarbonyl)pentylammonium-5-(dodecyloxycarbonyl)pentylcarbamate (T12). Method A: Neat DDEAC was placed into a CO_2 atmosphere for 2 h. The yellowish crystalline product was recrystallized from toluene (at dissolving T12 in toluene, the temperature cannot exceed 50°C!). Method B: CO_2 was introduced into an ethereal solution of DDEAC for 0.5 h. The crystalline product was filtered, washed with diethylether, and dried *in vacuo* at room temperature. MW = 643.0 g/mol. Yield: 85% (A)–89% (B). White crystals, mp = 62–65°C. IR (KBr): ν_{max} 3360, 1734, 1735, 1650, 1617 cm^{-1}; ^1H NMR (300 MHz, 70 mg of T12/0.7 ml $CDCl_3$, 5 drops of dry pyridine): δ 7.09 (3H; bs; NH_3^+); 4.79 (1H; bs; NH); 3.95 (4H; t; J = 6.8 Hz; $2OCH_2$); 3.05–2.90 (2H; m; CH_2NHCOO); 2.80–2.55 (2H; m; $CH_2NH_3^+$); 2.20 (4H; t; J = 7.4 Hz; $2CH_2CO$); 1.60–1.05 (52H; m; $26CH_2$); 0.78 (6H; t; J = 6.6 Hz; $2CH_3$); ^{13}C NMR (75 MHz, 70 mg of T12/0.7 ml $CDCl_3$, 5 drops of dry pyridine): δ 173.53 (COO); 161.98 ($NHCOO^-$); 64.20; 41.02; 40.50; 34.06; 31.71; 30.04; 29.43; 29.38; 29.33; 29.14; 29.06; 28.44; 26.27; 25.72; 24.55; 22.47; 13.89 ppm; −FAB: m/z 342.2 $[RNHCOO]^-$, 298.2 $[RNH]^-$; +FAB: m/z 300.2 $[RNH_3]^+$ [R = $C_{12}H_{25}OOC(CH_2)_5$]; Anal. ($C_{37}H_{74}N_2O_4$) C, H, N.

Skin Preparation

Human cadaver skin was purchased from the Tissue Bank of the Teaching Hospital in Hradec Králové, Czech Republic. The skin grafts from identical areas on a thigh from donors of either sex (60% female; with mean age 66 ± 9 years) were collected under aseptic conditions using a dermatome to gain samples of a thickness of about 300 μm. The skin samples were maintained in a wet chamber, and then adjusted into polyethylene bags, which were evacuated, closed, and stored at −20°C.

Permeation Experiments

The permeation-enhancing activities of T12 and DDEAC were evaluated *in vitro* using the Franz diffusion cells (17). The skin samples were slowly thawed (at 4°C first and then at ambient temperature), cut into pieces, and mounted into the cells dermal side down to leave a diffusion area of 2 cm^2. Closely adjacent skin pieces cut from each graft were used for the permeations with the control samples

and the pertinent enhancer. The acceptor compartment of the cell was filled with the pertinent acceptor phase (Table I) and allowed to equilibrate in a 32°C water bath with stirring for 30 min. The precise volume of the acceptor compartment (16–18 ml) was measured for each cell and was included into the calculations. The donor sample of 750-μl volume was applied on the skin surface, and the donor compartment of the cell was occluded with a cover glass. Samples of the acceptor phase of 0.6-ml volume were withdrawn at predetermined intervals over 48 h and were replaced with fresh acceptor phase.

Donor Samples

The donor samples were prepared by dispersing the pertinent drug in a given vehicle (see Table I for details). The samples with the enhancers were prepared by first dispersing the enhancer (1%) in the vehicle and then adding the drug. At this concentration, T12 was suspended, with partial dissolution. The suspensions were stirred for 5 min at 45°C (except for griseofulvin, which was dispersed at room temperature), allowed to equilibrate at 37°C for 24 h, and redispersed before application on the skin.

To evaluate the effect of DDEAC in the form of a pure amino ester, the substance was dissolved in $CHCl_3$ to cleave the carbamate salt that could have been formed during handling, and the solvent was evaporated under a stream of argon. A vehicle flushed with argon was added to the liquid enhancer, and the mixture was stirred at 45°C for 5 min. Theophylline was subsequently dispersed, and a stream of argon was bubbled through the mixture for several minutes to exclude the presence of carbon dioxide. The donor compartment of the cell was prefilled with argon and, after the application of the DDEAC-containing sample, covered to prevent the reaction with air carbon dioxide and the formation of the carbamate salt during the permeation experiment.

All donor samples were realized to be saturated because they were partly overloaded by the drug as well as the enhancer at the given conditions. The addition of the enhancers very probably had an effect on the solubility of drug and *vice versa*, but thermodynamic activity of both of them within the donor samples was realized to be maximal under the whole permeation experiment. The reported pH values were adjusted under a pH-metric control and checked prior the application, so the pH values of the samples applied to the skin were the same for both the enhancer-containing and control samples.

HPLC Determination of the Model Drugs

High-performance liquid chromatography analyses were performed using an ECOM LCP high-pressure pump, ECOM autosampler, Spectra Physics 8440 UV detector, and CSW 1.7 integrating software (Prague, Czech Republic). The samples of the acceptor phase were injected into the column without further treatment.

The amount of theophylline in the acceptor phase samples was determined using a LiChroCART 250-4 column (LiChrospher 100, RP 18, 5 μm) and methanol/0.1 M NaH_2PO_4 4:6 v/v as the mobile phase at a flow rate of 1.2 ml/min. The detector wavelength was set at 272 nm. The retention time of theophylline was 3.3 min.

Clotrimazole was determined using a LiChroCART 250-4 column (LiChrospher 60 RP-Select B, 5 μm) and methanol/0.025 M K_2HPO_4 5:1 v/v as the mobile phase at a flow rate of 1.2 ml/min. The effluent was monitored at 227 nm, and the retention time of clotrimazole was 4.2 min.

Flobufen was determined using a LiChroCART 125-4 column (LiChrospher 100, RP 18, 5 μm) and acetonitrile/phosphate buffer at pH 3.0, 6:4 v/v, as the mobile phase at a flow rate of 1.2 ml/min. The effluent was monitored at 279 nm, and the retention time of flobufen was 2.5 min.

Griseofulvin was determined using a LiChroCART 125-4 column (LiChrospher 100, RP 18, 5 μm) and methanol/water 7:3 v/v as the mobile phase at a flow rate of 1.0 ml/min. The effluent was monitored at 291 nm, and the retention time of griseofulvin was 2.3 min.

Data Treatment

The cumulative amount of the drug having penetrated the skin, corrected for the acceptor sample replacement, was plotted against time. The steady-state flux ($\mu g/cm^2/h$) was calculated from the linear region of the plot. The enhance-

Table I. The Properties of the Model Drugs and the Composition of the Donor Samples and Acceptor Phases Used for the Permeation Experiments

Model drug	Theophylline	Clotrimazole	Flobufen	Griseofulvin
Physicochemical properties				
MW (g/mol)	180	345	304	353
log P[a]	0.05	5.8	3.8	2.4
pK_a[a]	8.6	6.1	4.5	–
mp (°C)	270–274[b]	147–149[b]	160–161[c]	220[b]
Donor sample				
Drug amount (%)	2.5	1.0	1.0	0.1
Vehicle, pH	w	t7.3/PG/E 5:4:1	t7.0/PG 1:1	w/PG 2:3
	t7.3	t8.3/PG/E 5:4:1	t8.0/PG 1:1	
	t8.3	t9.3/PG/E 5:4:1	t8.7/PG 1:1	
Acceptor phase	PBS 7.4	PBS 7.2/PG/E 5:4:1	PBS 6.6/PG 1:1	PBS 7.4/E 6:4

w = water; t7.3 = Tris buffer at pH 7.3, etc.; PG = propylene glycol; E = ethanol; PBS = phosphate-buffered saline with 0.03% NaN_3.
[a] Chemical Abstracts; calculated by Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67.
[b] The Merck Index, Merck & Co., Inc., Whitehouse Station, NJ, USA.
[c] See Kuchar *et al.* (16).

ment ratio (ER) value was calculated as the ratio of the flux of the drug with an enhancer and the flux of the drug alone.

The data are presented as means ± SD obtained using the skin samples from at least three donors. The statistical significance of the differences between the drug permeation with and without the enhancer was analyzed using the Student's *t* test. A value of $p < 0.05$ was considered significant.

Enzymatic Hydrolysis

Freshly boiled water of 9.8-ml volume with 100 μl of 0.2% (w/v) T12 solution in acetonitrile (i.e., 200 μg of T12) was stirred in a water bath at 37°C for 10 min. Subsequently, 100 μl of a 1:1000 dispersion of porcine esterase in water (0.4 U of the enzyme) was added. At predetermined time intervals, 5 ml of acetonitrile was added to the reaction vessel to quench the hydrolysis. The mixture was carefully evaporated at 35°C and dried *in vacuo* over P_2O_5. The same reaction was carried out without addition of the enzyme.

The residue was dissolved in 5 ml of chloroform, and 20 mg of 3,5-dinitrobenzoyl chloride (DNBC) and 0.2 ml of triethylamine were added. The mixture was heated at reflux for 0.5 h, evaporated to dryness, and allowed to stand over H_2SO_4 *in vacuo* overnight to remove the excess triethylamine. The derivatized mixture was then dissolved in 1.0 ml of acetonitrile and analyzed by HPLC using a LiChroCART 125-4 column (LiChrospher 100, RP 18, 5 μm, Merck). The mobile phase consisted of a mixture of acetonitrile/water/acetic acid, 80:20:1, and a flow rate of 1.5 ml/min was used. DNB–T12 solution of 10-μl volume was injected onto the column and monitored at 230 nm, with the retention time of 9.3 ± 0.4 min. The calibration provided good linearity over the concentration range of 0.05–200 μg/ml.

The presence of dodecanol on the reaction was determined by thin-layer chromatography (TLC). The reaction mixture after 180 min was evaporated to dryness and analyzed on TLC plates (silica gel 60 F_{254}, aluminum back, Merck), using chloroform/methanol 9:1 mobile phase and standard T12 and dodecanol solutions. The spots were visualized by $Ce(SO_4)_2$, $H_3[P(Mo_3O_{10})_4]$, and H_2SO_4 at 180°C.

RESULTS

Synthesis of DDEAC and T12

Initially, DDEAC was synthesized, as described in our preceding article (11). IR spectra of the product measured in a KBr tablet showed that the substance contained, at least in part, an ammonium carbamate salt. Thus, the process formerly described as crystallization was actually a reaction of the amino ester DDEAC with carbon dioxide, yielding the crystalline ammonium carbamate salt T12. Therefore, the term DDEAC was previously used incorrectly, and, in this article, it is used purely for the amino ester, whereas the carbamate is termed T12.

To confirm the structure of T12 and to compare its effect with DDEAC, both substances were prepared. Because of high affinity of DDEAC to carbon dioxide, it was not possible to isolate the pure amino ester using the previously described procedure (11). Moreover, a relatively fast intramolecular cyclization of DDEAC to ε-caprolactam was observed. Therefore, it was more convenient to prepare DDEAC by the degradation of the carbamate immediately before permeation experiments. Pure T12 can be prepared either by reaction of the liquid amino ester with CO_2 or by introducing CO_2 into its ethereal solution. The latter method yields an easy-to-isolate product of high purity.

Confirmation of the Structure of T12

Infrared spectra of T12 in a KBr tablet showed a strong carbamate carbonyl stretching vibration at 1617 cm^{-1} with a shoulder at 1650 cm^{-1}, and a doublet of ester carbonyl vibration at 1735 and 1743 cm^{-1}, indicating two ester groups with different hydrogen bonding. Similar results were obtained in Nujol suspension and using attenuated total reflection crystal. However, when recorded in a $CHCl_3$ solution, no such vibration was observed. For a detailed IR and Raman spectroscopic study of T12, see Zbytovská *et al.* (15).

For the NMR spectroscopy, a suitable solvent that dissolves but not decomposes the carbamate salt was needed. Because of a very low solubility of T12 in most organic solvents, the NMR spectra were recorded in $CDCl_3$ saturated with dry pyridine with high concentration of the sample (10% w/v). The ^{13}C NMR spectrum showed a signal at 161.98 ppm, attributed to the carbonyl carbon of the carbamate salt, and two resonances at 41.02 and 40.50 ppm, corresponding to the methylene carbons next to NH_3^+ and $NHCOO^-$, respectively. The ^1H-NMR spectrum further confirmed the carbamate structure; two signals of the methylene hydrogens adjacent to ammonium and carbamate nitrogen were present at the ratio of 1:1.

FAB was used for the mass spectrometric characterization of T12. Positive FAB showed an ion at *m/z* 300.2, which corresponds to the ammonium salt $C_{12}H_{25}OOC(CH_2)_5NH_3^+$. In the negative FAB spectrum, the compound showed a strong *m/z* 342.2 ion, corresponding to the carbamate anion $C_{12}H_{25}OOC(CH_2)_5NHCOO^-$ together with a weaker ion at *m/z* 298.2 (loss of CO_2). Furthermore, CHN analysis results were in accordance with the carbamate structure.

Permeation Experiments

Flux and ER values for the permeations of theophylline, clotrimazole, flobufen, and griseofulvin are summarized in Table II. Typical permeation profiles for each drug are shown in Fig. 2. Theophylline flux from both buffers and nonbuffered aqueous vehicle was approximately 3.5 μg/cm^2/h. While T12 exhibited high enhancing activity for theophylline from all tested donor vehicles (with fluxes of approximately 170 μg/cm^2/h, and the corresponding ER values up to 55.6), DDEAC was completely inactive.

The data in Table IIb show clotrimazole permeation from vehicles at different pH in the presence of T12. The flux values slightly increased with increasing pH of the donor sample up to 1.0 μg/cm^2/h. The addition of 1% of T12 increased the permeation 7.0- and 7.7-fold at pH 7.3 and 8.3, respectively; however, it had a very little effect at pH 9.3.

The data for flobufen permeation are listed in Table IIc. The flux values without an enhancer were approximately 1 μg/cm^2/h; the addition of T12 led to flux values of 4.6, 4.0, and 2.9 μg/cm^2/h at pH 7.0, 8.0, and 8.7, respectively.

Table II. Permeation Characteristics of the Model Drugs and the Enhancing Effect of 1% DDEAC and T12, Respectively

Vehicle/enhancer	Flux ± SD (μg/cm^2/h)	ER
a. Theophylline		
w	3.8 ± 1.0	
w/T12	166.6 ± 34.0[a]	43.3
w/DDEAC	3.6 ± 0.7	0.9
t7.3	3.5 ± 1.9	
t7.3/T12	172.2 ± 75.4[a]	49.2
t7.3/DDEAC	4.9 ± 1.9	1.4
t8.3	3.2 ± 1.3	
t8.3/T12	176.7 ± 78.7[a]	55.6
t8.3/DDEAC	4.2 ± 0.6	1.3
b. Clotrimazole		
t7.3/PG/E	0.5 ± 0.1	
t7.3/PG/E/T12	3.3 ± 0.4[a]	7.0
t8.3/PG/E	0.6 ± 0.1	
t8.3/PG/E/T12	3.9 ± 0.8[a]	7.7
t9.3/PG/E	1.0 ± 0.3	
t9.3/PG/E/T12	1.4 ± 0.3	1.4
c. Flobufen		
t7.0/PG	0.9 ± 0.2	
t7.0/PG/T12	4.6 ± 1.0[a]	5.0
t8.0/PG	1.1 ± 0.4	
t8.0/PG/T12	4.1 ± 1.2[a]	3.9
t8.7/PG	1.5 ± 0.5	
t8.7/PG/T12	2.9 ± 1.1	1.9
d. Griseofulvin		
w/PG	<0.17[b]	
w/PG/T12	4.1 ± 0.8[a]	>24[b]

n = 4–8
w = water; t7.3 = Tris buffer at pH 7.3, etc.; PG = propylene glycol; E = ethanol; DDEAC = dodecyl-6-aminohexanoate; T12 = Transkarbam 12; ER = enhancement ratio.
[a] Significantly different from control ($p < 0.05$).
[b] In two control experiments, no griseofulvin was detected in the acceptor samples. These data were excluded from the calculation of the mean flux; consequently, the actual value is lower and the corresponding ER is higher.

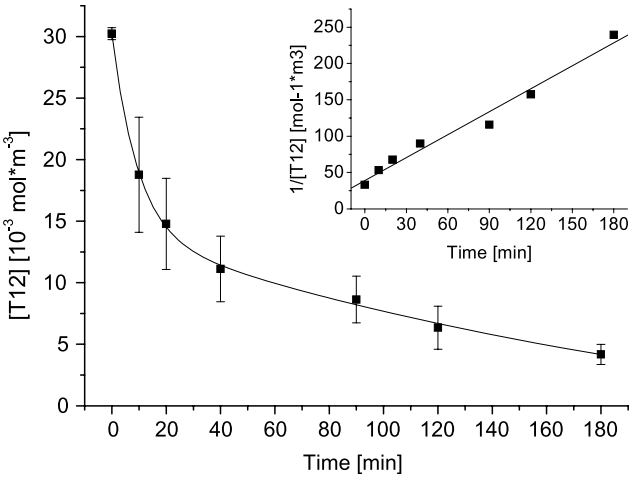

Fig. 3. *In vitro* hydrolysis of T12 in the presence of porcine esterase (second-order reaction). T12 concentration [T12] = $-4.9 + 16.2 \times e^{-t/9.4} + 19.0 \times e^{-t0/244}$; $R^2 = 0.99988$. Insert: plot of 1/[T12] against time, $p < 0.0001$; $k = 0.0175$ mol^{-1} m^3 s^{-1}; $t_{1/2} = 31$ min.

Flux and ER values for griseofulvin permeation are shown in Table IId. In two control experiments, no griseofulvin was detected in the acceptor samples, and these experiments were excluded from the calculation of the mean flux and, consequently, the ER value and t tests. Therefore, the real flux is likely to be lower and the ER value higher. The addition of 1% of T12 to the donor sample, however, resulted in a reproducible flux value of 4.1 μg/cm^2/h.

Enzymatic Hydrolysis of T12

Figure 3 shows a plot of T12 concentration [T12] *vs.* time in the presence of porcine esterase. The degradation of the ester bond followed a second-order kinetics with the apparent second-order rate constant $k = 0.0175$ mol^{-1} m^3 s^{-1} and the estimated half-life $t_{1/2}$ having been approximately 31 min. No decrease in T12 concentration was observed in the same experiment without addition of the esterase, which excludes chemical hydrolysis. For the determination of T12, a reversed-phase HPLC method was developed. T12 was converted into DDEAC and derivatized by DNBC for

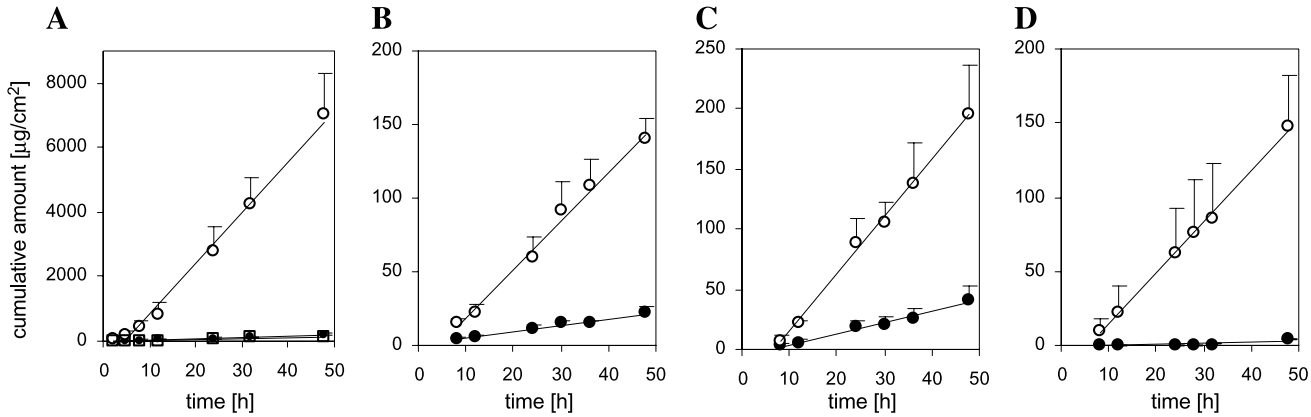

Fig. 2. Examples of the permeation profiles of the model drugs through the human skin from a control sample (•), and in the presence of 1% T12 (○) and 1% DDEAC (□), respectively. Drug, vehicle: (A) theophylline, w, (B) clotrimazole, t7.3/PG/E, (C) flobufen, t7.0/PG, (D) griseofulvin, w/PG.

ultraviolet detection. The detection limit was 50 ng/ml. As this method describes only decomposition of T12 and not the appearance of metabolites, the presence of the dodecanol has been confirmed by TLC (chloroform/methanol 9:1, Rf values for dodecanol and T12 were 0.7 and 0.1, respectively).

DISCUSSION

Skin permeation enhancer DDEAC was designed as an acyclic Azone analog. Because IR and NMR spectra in chloroform confirmed the amino ester structure in the previous study, we believed that opening of the azepan-2-one cycle of Azone and substitution of the amide with an ester group were the reasons of the markedly increased activity of this novel enhancer (11). However, this substance showed a crystalline structure with relatively high melting point and was insoluble in a number of organic solvents, which was unusual of the suggested amino ester structure. Moreover, an acidimetric titration showed that the substance contained only 91% of DDEAC.

Thus, it was suggested that the structure of the enhancer is actually an alkylammonium salt of an alkylcarbamic acid—T12—produced by the reaction of DDEAC with carbon dioxide. The formation of carbamates from ammonia and primary and secondary amines is a well-known reaction of great importance in biology and industrial applications. For an extensive review on carbamate derivatives, see Dell'Amico *et al.* (18). A similar carbamate structure was found in skin permeation enhancers, based on tranexamic acid (*trans*-4-aminomethylcyclohexanecarboxylic acid) derivatives (19).

In this study, the carbamate structure of the enhancer was fully confirmed by spectral methods. Therefore, the activity and toxicity data reported previously apply to the carbamate salt, i.e., T12 (11). The reason why the IR and NMR spectra confirmed the amino ester structure in the previous study is the reversibility of the reaction. T12 can decompose into DDEAC and carbon dioxide in an acidic environment or upon heating (15). It was found during this work that trace amounts of protons in chloroform were able to decompose the carbamate; therefore, the previously measured spectra were consistent with the structure of DDEAC. This chloroform-mediated carbamate cleavage was used in the preparation of pure DDEAC.

Previously investigated thermotropic behavior of T12 showed the first transition at about 53°C, related to a change in the carbamate polar head (15). This explains why CHN analysis had confirmed DDEAC structure (11), as the sample for the analysis was dried *in vacuo* at 54°C. Although CO_2 stays connected to the molecule via a noncovalent interaction and is not being released during this transition (15), the vacuum could shift the equilibrium toward DDEAC by removing the loosely bound CO_2. The analysis of a sample, dried at ambient temperature, was in accordance with the structure of T12.

As already noted, T12 can be decomposed into DDEAC in a slightly acidic environment. As stratum corneum, the site of action of permeation enhancers, is of acidic nature, the decomposition of T12 into DDEAC could be expected. Therefore, we aimed at identifying the nature of the actual active substance, i.e., either T12 or the released DDEAC.

The enhancement activities of T12 and DDEAC were compared using the Franz diffusion cell, human skin, and theophylline as a model drug, i.e., under the same conditions as in the previous study (11). Theophylline is a weak base of medium lipophilicity (log $P \sim 0$) and a small molecular weight (Table I). The activity of DDEAC was evaluated under argon, including sample preparation, to avoid its reaction with carbon dioxide. This enabled us to compare directly the enhancement ability of the free amino ester DDEAC and its corresponding ammonium carbamate salt T12. The results demonstrated that only the carbamate structure was responsible for the observed enhancing effect. The reason for the striking difference in the activities of these compounds remains unclear and is currently under investigation. It could be connected with the following:

1. The unusual structure of the polar head, resulting in a specific interaction with the stratum corneum components
2. The lability of the carbamate bond in an acidic environment and a consequent carbon dioxide release in the stratum corneum
3. The presence of two hydrophobic chains in the enhancer, which is similar to ceramides and could lead to a better incorporation of the enhancer into a lipid lamella
4. A combination of some or all the three reasons given above

To evaluate the permeation-enhancing properties of T12 in greater detail, additional three drugs, including clotrimazole, flobufen, and griseofulvin, covering a wide range of physicochemical properties, were included in the study (Table I). Substances of a molecular weight up to 353 g/mol were selected because this weight is similar to that of oxybutynin, currently the largest drug incorporated in a patch (20). The polarity of the drugs ranges from log $P \sim 0$ to 5.8; that is, their partition coefficients differ by five orders of magnitude. The melting points of the drugs vary between 147 and 274°C, and the compounds are of acidic, basic, and neutral nature.

Clotrimazole is an imidazole antifungal of a relatively high molecular weight and lipophilicity. The addition of 1% T12 enhanced the permeation of clotrimazole at pH 7.3 and 8.3; however, at pH 9.3, its effect was suppressed. A possible explanation of such pH-dependent effect is that in aqueous media, equilibrium between a carbamate, bicarbonate, and carbonate occurs, and basic conditions would favor bicarbonate and/or carbonate formation (18). This implies that the alkylcarbamate anion is the most important structural feature for the enhancing effect of the molecule. However, the behavior of T12 under aqueous conditions and the pH dependence of the enhancement effect merit further investigation.

Flobufen is a novel anti-inflammatory drug with immunomodulatory effects (21). It has been included in the study as an example of a weak acid. Previously, T12 was inactive when coadministered with an acidic drug, most likely because of acid-catalyzed decomposition of the carbamate (unpublished observation). However, in a buffered donor vehicle, T12 enhanced the permeation of acidic flobufen as well as that of neutral and weakly basic drugs. Similar to clotrimazole permeation, a decreased enhancement was observed at pH 8.7, suggesting an increased bicarbonate/carbamate ratio.

Griseofulvin is an antifungal substance, produced by the growth of certain strains of *Penicillium griseofulvum*. In this study, it was selected as a representative of large, neutral, and poorly permeating substances. Using an aqueous receptor phase, no griseofulvin was detected. The addition of a relatively high amount of ethanol to the acceptor (40%) resulted in detectable permeation, although not in all experiments. The flux of griseofulvin without an enhancer was not further evaluated because the effect of T12 was clearly shown—an addition of the enhancer resulted in a reproducible flux of griseofulvin, which is usually concentrated in the stratum corneum and does not penetrate into lower layers.

The potential of T12 to be enzymatically hydrolyzed *in vitro* was shown using porcine esterase. As esterases are present in the human epidermis (22), the hydrolysis is likely to occur *in vivo* as well. Such hydrolysis in the viable epidermis would prevent the action of the enhancer on the living cells. The metabolites, e.g., 6-aminohexanoic acid and dodecanol, are compounds of very low toxicity, which is another favorable characteristic of this novel enhancer. Furthermore, the described HPLC method for T12 determination could be useful in further evaluation of its effect.

Furthermore, toxicity of T12 has been newly evaluated. Repeated daily dose 28-day dermal toxicity study in rat with a 14-day treatment-free period revealed no significant clinical symptoms including site of administration in a group receiving 0.1% T12, a slight skin erythema at 2% T12, and a skin erythema and crusts at 10% concentration. After the end of administration, skin recovered during 5–14 days. Significant changes in total leukocyte count were observed, which correlated with the skin reaction. No changes in body weight, food consumption, organ weights, red blood cell parameters, serum chemistry parameters, and no hepato- and nephrotoxicity have been found in all concentrations used (23). No toxicity signs were observed in rats in the acute oral toxicity test in doses of 50, 300, and 2000 mg/kg, neither during 24 h after administration nor during 14 days of observation period (24). Index of dermal irritation was 0.25 (category nonirritating) and 1.67 (category slightly irritating) for 0.5 and 5% suspension of T12 in PG, respectively (25). T12 did not act as a contact allergen in the closed patch sensitization test (Buehler's method) (26). Moreover, T12 had no mutagenic potential in bacteria (the Ames reverse mutation test) (27) and rats and no cytotoxic effect on the bone marrow of rats (micronucleus test) (28). All tests were performed in accordance with the pertinent OECD Guidelines for Testing of Chemicals.

CONCLUSION

In this study, the properties of a promising transdermal permeation enhancer T12 have been investigated. Comparing both physicochemical and permeation-enhancing properties clearly showed that the carbamate salt, not the parent amino ester DDEAC, was responsible for the high activity of the compound. The activity of T12 was demonstrated using four model drugs of different physicochemical properties, and the susceptibility of the compound to metabolic deactivation into nontoxic substances was shown *in vitro* using porcine esterase.

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REFERENCES

1. A. C. Williams and B. W. Barry. Penetration enhancers. *Adv. Drug Deliv. Rev.* **56**:603–618 (2004).
2. R. B. Stoughton. Enhanced percutaneous penetration with 1-dodecylazacycloheptan-2-one. *Arch. Dermatol.* **118**:474–477 (1982).
3. J. W. Wiechers and R. A. DeZeeuw. Transdermal drug delivery: efficacy and potential applications of the penetration enhancer Azone®. *Drug Des. Deliv.* **6**:87–100 (1990).
4. N. Büyüktimkin, S. Büyüktimkin, and J. H. Rytting. Chemical means of transdermal drug permeation enhancement. In T. K. Ghosh, W. R. Pfister (eds.), *Transdermal and Topical Drug Delivery Systems*, Interpharm Press, Buffalo Grove, IL, 1997, pp. 357–476.
5. B. B. Michniak, M. R. Player, J. M. Chapman, and J. W. Sowell Sr. *In vitro* evaluation of a series of Azone analogs as dermal penetration enhancers. I. *Int. J. Pharm.* **91**:85–93 (1993).
6. B. B. Michniak, M. R. Player, L. C. Fuhrman, C. A. Christensen, J. M. Chapman, and J. W. Sowell Sr. *In vitro* evaluation of a series of azone analogs as dermal penetration enhancers: III. Acyclic amides. *Int. J. Pharm.* **110**:231–239 (1994).
7. B. B. Michniak, M. R. Player, D. A. Godwin, C. A. Phillips, and J. W. Sowell Sr. *In vitro* evaluation of a series of Azone analogs as dermal penetration enhancers: IV. Amines. *Int. J. Pharm.* **116**:201–209 (1995).
8. B. B. Michniak, M. R. Player, and J. W. Sowell Sr. Synthesis and *in vitro* transdermal penetration enhancing activity of lactam N-acetic acid esters. *J. Pharm. Sci.* **85**:150–154 (1996).
9. L. C. Fuhrman Jr., B. B. Michniak, C. R. Behl, and A. W. Malick. Effect of novel penetration enhancers on the transdermal delivery of hydrocortisone: an *in vitro* species comparison. *J. Control. Release* **45**:199–206 (1997).
10. K. Vávrová, J. Zbytovská, and A. Hrabálek. Amphiphilic transdermal permeation enhancers; structure–activity relationships. *Curr. Med. Chem.* **12**:2273–2291 (2005).
11. P. Doležal, A. Hrabálek, and V. Semecký. epsilon-Aminocaproic acid esters as transdermal penetration enhancing agents. *Pharm. Res.* **10**:1015–1019 (1993).
12. A. Hrabálek, P. Doležal, O. Farsa, A. Krebs, A. Kroutil, M. Roman, and Z. Šklubalová. ω-Amino acid derivatives, processes of their preparation and their use. U.S. Pat. 6,187,938 (2001).
13. R. Ibuki. Ph.D. Thesis. University of Kansas, Lawrence, 1985.
14. A. Hrabálek, P. Doležal, and K. Palát. Physico-chemical parameters and skin permeation enhancing effect of ω-amino acid derivatives. In K. R. Brain, K. A. Walters (eds.), *Perspectives in Percutaneous Penetration, Vol 7A*, STS Publishing, Cardiff, 2000, p. 70.
15. J. Zbytovská, S. Raudenkolb, S. Wartewig, W. Hübner, W. Rettig, P. Pissis, A. Hrabálek, P. Doležal, and R. Neubert. Phase behaviour of transkarbam 12. *Chem. Phys. Lipids* **129**:97–109 (2004).
16. M. Kuchar, M. Poppova, H. Zunova, E. Knezova, V. Vosatka, and M. Prihoda. 4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid and its derivatives. *Collect. Czechoslov. Chem. Commun.* **59**:2705–2713 (1994).
17. T. J. Franz. Percutaneous absorption; on the relevance of *in vitro* data. *J. Invest. Dermatol.* **64**:190–195 (1975).

18. D. B. Dell'Amico, F. Calderazzo, L. Labella, F. Marchetti, and G. Pampaloni. Converting carbon dioxide into carbamato derivatives. *Chem. Rev.* **103**:3857–3898 (2003).
19. K. Vávrová, A. Hrabálek, P. Doležal, T. Holas, and J. Klimentová. Biodegradable derivatives of tranexamic acid as transdermal permeation enhancers. *J. Control. Release* **104** 41–49 (2005).
20. M. R. Prausnitz, S. Mitragotri, and R. Langer. Current status and future potential of transdermal drug delivery. *Nat. Rev., Drug Discov.* **3**:115–124 (2004).
21. V. Panajotová, E. Anděrová, A. Jandera, and M. Kuchař. Pharmacological profile of the novel antirheumatic 4-(2′,4′-difluorobiphenyl)-2-methyl-4-oxobutanoic acid. *Arzneimittelforschung.* **47**:648–652 (1997).
22. W. Montagna. Histology and cytochemistry of human skin IX. The distribution of non-specific esterases. *J. Biophys. Biochem. Cytol.* **1**:13–16 (1955).
23. Report No. 031/04/L. Repeated Dose 28-Day Dermal Toxicity Study in Rat with a 14-Day Treatment-Free Period. In accordance with OECD Guidelines for Testing of Chemicals No. 410. Bochemie Ltd., Bohumín, Czech Republic (2005).
24. Report No. 017/04/L. Acute Oral Toxicity in Rats, Acute Toxic Class Method. In accordance with OECD Guidelines for Testing of Chemicals No. 423. Bochemie Ltd., Bohumín, Czech Republic (2004).
25. Report No. 025/04/L. Acute Dermal Irritation/Corrosion—Rabbit. In accordance with OECD Guidelines for Testing of Chemicals No. 404. Bochemie Ltd., Bohumín, Czech Republic (2004).
26. Report No. 024/04/L. Skin Sensitisation in Guinea Pigs. In accordance with OECD Guidelines for Testing of Chemicals No. 406. Bochemie Ltd., Bohumín, Czech Republic (2004).
27. Report No. 027/04/L. Bacterial Reverse Mutation Test. In accordance with OECD Guidelines for Testing of Chemicals No. 471. Bochemie Ltd., Bohumín, Czech Republic (2004).
28. Report No. 023/04/L. Micronucleus Test in Rats. In accordance with OECD Guidelines for Testing of Chemicals No. 474. Bochemie Ltd., Bohumín, Czech Republic (2004).