Permeability Profiles of M-Alkoxysubstituted Pyrrolidinoethylesters of Phenylcarbamic Acid across Caco-2 Monolayers and Human Skin

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Purpose. The purpose of the present research was to study 10 m-alkoxysubstituted pyrrolidinoethylesters of phenylcarbamic acid—potential local anesthetics. The relationships between the structure of the molecule, its physicochemical parameters (log D_{oct}, log k, R_M, solubility) were correlated to the permeability data obtained from permeation experiments in Caco-2 monolayers and excised human skin *in vitro*.

Methods. The extent and mechanism(s) of permeability of the series were studied through a Caco-2 monolayer in the apical-to-basolateral (a-b) and basolateral-to-apical (b-a) directions. The MTT test was performed to determine cellular damage. *In vitro* transdermal permeability data were obtained from permeation experiments on excised human skin by using side-by-side chambers. Passive diffusion and iontophoretically enhanced permeability were measured.

Results. In Caco-2 monolayers, similar results in the shape of the permeability curves were obtained for the two directions. In the b-a direction, the values of P_{app} were ~2–6 times greater than in the a-b direction. A plot of drug permeability vs. the number of carbons in the alkoxychain plateaued first, after which the permeability decreased by the increasing lipophilicity of the drug. If the log D_{oct} of the ester was $\geq$ 3.4 and the MW > 385 Da, no measurable Caco-2 permeability was found. Cell damage was also higher by the more lipophilic compounds. In excised human skin, the relationship between the passive diffusion of the drugs and the number of carbons in the alkoxychain was parabolic ($r^2 = 0.95$). Introducing low-level electrical current (iontophoresis), transdermal permeability of the more hydrophilic phenylcarbamic acid esters increased clearly.

Conclusions. Lipophilicity and solubility of a compound have crucial roles in the permeation process. A very high lipophilicity has, however, a negative influence on the permeability, both intestinally and transdermally. Iontophoresis significantly increases the diffusion of small and less lipophilic compounds.

KEY WORDS: drug permeability; lipophilicity; phenyl carbamic acid esters; Caco-2; human skin.

INTRODUCTION

Successful drug development requires not only the optimization of specific and potent pharmacologic activity at the target site but also efficient delivery to that site, *i.e.,* clinical development is often stymied by delivery problems (1). To improve the drug development and reduce pharmacologic testing of new "useless" compounds, consideration of the lipophilicity-hydrophilicity balance and its correlation with permeability may be helpful. Several physicochemical parameters, *e.g.,* molecular weight/volume, partition/distribution coefficient, hydrogen bond donor/acceptor capacity, and dynamic polar surface area, have proved particularly useful for predicting drug permeation across biomembranes such as gut wall/Caco-2 cell lines (2–5) or human skin (6–8). The attribute that has attracted the most interest in QSAR studies is, of course, the lipophilicity of a compound. The most common expression of lipophilicity is the logarithm of the n-octanol/water partition (distribution) coefficient log P_{oct} (log D_{oct}). Log P_{oct} is also a useful parameter for correlating the transport processes of a drug into its interaction with a receptor (9).

Because ~90% of all the marketed drugs are administered orally, the principal physiologic barrier the drugs have to pass to enter the body is the intestinal mucosa (10). Caco-2 monolayers have been shown to form well-differentiated monolayers that possess very similar properties as the enterocytes of the small intestine. Caco-2 cell monolayers are often used as an *in vitro* model for epithelial drug uptake (4,5,11), and they have been accepted as a method to waive bioavailability/bioequivalence testing required by the FDA in certain classes of biopharmaceuticals (12). An optional route for drugs to enter the body is skin membrane. To enhance the transdermal transport of drugs, chemical and/or physical permeation enhancement is often needed (13). Iontophoresis (*i.e.,* imposed electric field across the membrane) is one way to achieve improved local and systemic drug effect. The lipophilicity of a drug has a significant influence on transdermal delivery via both the passive diffusion and iontophoresis (6–8,14,15).

The model compounds used in this correlation study were a synthesized homologous series of m-alkoxysubstituted pyrrolidinoethylesters of phenylcarbamic acid (Fig. 1). Their chemical structure can be modified in all parts of the molecule: in the lipophilic alkoxy ring, in the hydrophilic pyrrolidino part, and in the connective chain (16). Modification of the structure, obviously, influences the physicochemical and biological properties of the compounds. The intended use of these pharmacologically active agents is local anesthesia; therefore, the interest of (trans)dermal permeability of these compounds is obvious. The meta-substituent of the alkoxy ring has been shown to possess the highest correlation coefficients between the chemical structure and the physicochemical parameters, compared with orto- and para-substituted phenyl carbamic acids (17). Typically, for the compounds that have a common parent structure and differ from each other only in the alkyl chain length, a linear change in the physicochemical properties is expected. Thus, the purpose of the present research was to study the relationships between the structure of the molecule, its physicochemical parameters (log D_{oct}, log k, R_M, solubility, molecular weight), and the permeability data obtained from the permeation experiments in Caco-2 monolayers and excised human skin *in vitro*.

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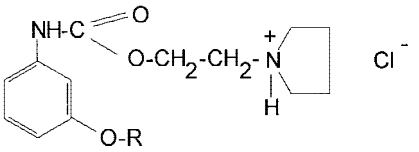

Fig. 1. Structure of the phenylcarbamic acid esters studied. R = C_nH_{2n+1}, when n = 1–10. In compound II the n = 1, and in V–XXIX the n = 2–10, respectively (see also Table I).

MATERIALS AND METHODS

Materials

The model drugs studied, pyrrolidinoethylesters of m-alkoxysubstituted phenylcarbamic acid (Fig. 1), were synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University, Slovakia, according to the literature (16). Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD) at passage 19. Dulbecco's modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS, heat inactivated at 56°C for 30 min), L–glutamine (200 mM), antibiotic mixture (1000 IU/mL penicillin G, 1000 μL/mL streptomycin) and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco BRL, Scotland. ^{14}C-labeled mannitol (specific activity = 51.50 μCi/mmol) as a marker of membrane integrity was obtained from DuPont NEN (Boston, MA). For the transdermal permeation studies, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and sodium chloride were from Sigma Chemicals Co. (St. Louis, MO). Agar was purchased from Tamro (Finland). D(-)-mannitol was from Merck (Darmstadt, Germany). Silver chloride for electrode preparation was also from Sigma. Deionized water (milli-Q, Millipore, Molsheim, France) was used to prepare all solutions. Excised human epidermis was used as a membrane. Skin samples were obtained from the Department of Pathology, University of Helsinki. Epidermis was separated and used in the diffusion experiments (14,15).

Caco-2 Transport Studies

Caco-2 monolayers were grown in DMEM containing 10% FBS, 1% NEAA, 1% L-glutamine, and 1% antibiotic solution in culture flasks in an incubator (BB 16-glass incubator; Heraeus Instruments GmbH, Germany). The cultures were kept at 37°C in an atmosphere of 5% CO_2, 95% air, and 95% relative humidity. The cell suspension (0.5 mL) with a density of 150,000 cells/mL was added to the apical side of the porous polycarbonate filter membranes with a pore size of 0.4 μm and a surface area of 1.1 cm^2 in clusters with 12 wells (Transwell, Costar, USA). The cells were given fresh medium three times a week until the time of use. Monolayers used for transport studies were between the 21st and 28th day on filters. Cells at passage 31–41 were used in all the studies. Just before the experiments, the culture medium was replaced with Dulbecco's PBS containing 5 mM $NaHCO_3$, and the monolayers were washed twice with this solution. The washing solution was replaced by Dulbecco's PBS containing 10 mM HEPES (pH 7.4). Transepithelial electrical resistance (TEER) of all the wells was measured before the start of each experiment by using Millicell® ERS (Millipore, USA) to ensure the integrity of the monolayers formed on the filters. The average TEER-value was between 350 and 550 ohms × cm^2.

Dulbecco's PBS containing 0.25 mM of the drug (pH = 7.4) was added to the apical (0.5 mL) or basolateral (1.5 mL) compartment. Samples were collected every 15 min for 2 h by moving the cell inserts to a new well with fresh medium (apical-to-basolateral direction) or by emptying the apical compartment by pipetting 0.5 mL (the whole volume of the apical compartment) and adding new medium (basolateral-to-apical direction). The samples were analyzed by HPLC. The monolayer integrity was assessed by using the radioactive ^{14}C-mannitol alone or with the drug. For the monolayer integrity, the permeation rate of mannitol should not exceed 0.5% per hour.

MTT Assay

MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenases of living cells to give a dark-blue formazan product (18). Damaged or dead cells show reduced or no dehydrogenase activity. Caco-2 cells were seeded in 96-well tissue culture plates (Costar) and incubated for 22–24 h at 37°C. A cell concentration of 50,000 cells/well was used (18). The cells were incubated for 60 min with drugs (0.25 mM, rows 1–10) or with HEPES-buffered saline (row 11, which contained cells = negative control); row 12 did not contain cells (= background). Thereafter, the MTT solution (5 mg/mL in Dulbecco's PBS) was added, and the plates were incubated for another 90 min. The solution was removed, and the formazan crystals in the cells were dissolved by 200 μL of a solution consisting of 10% sodium dodecyl sulfate and 0.01 M HCl in isobutanol. The color was measured at λ = 590 nm in a multiwell scanning spectrophotometer (Model 550 Microplate Reader, Biorad, Japan). The cytotoxicity of the compounds was calculated as the percent of vital activity compared with the blank (blank = negative control – background).

Transdermal Permeation Studies

The *in vitro* permeation studies were performed in side-by-side chambers (Laborexin Inc., Helsinki, Finland), in which a piece of human skin separated the donor and acceptor chambers (1.1 mL). The available diffusion area between the chambers was 0.785 cm^2. The experiments (n = 3–6) were performed by seven drugs from the series studied (V–XXII in Table I). The donor cells, faced with the stratum corneum, contained a 10 mM solution of the tested drugs + 1 mM mannitol "spiked" with 5 μL/mL of the ^{14}C-radiolabeled sugar in 25 mM HEPES-buffer (pH = 7.4). The receptor cells were filled with blank buffer. At predetermined intervals (1, 2, 4, 8, 12, and 24 h), 300 μL of the receptor solution was withdrawn and refilled with the same volume of fresh buffer. For the iontophoretic permeation studies, silver-silver chloride electrodes were used (14). The power supply to generate the constant current between the electrodes (anode in the donor compartment and cathode in the acceptor compartment) was Ministat (Sycopel Scientific Inc., Washington, UK). The voltage and current were monitored continuously during the experiments. Constant current iontophoresis at 0.5 mA/cm^2 was carried out for 12 h, after which passive permeation was followed up to 24 h (15). Part of each sample (150

Table I. Physichochemical Properties of the Phenylcarbamic Acid Esters Studied

Label	R	MW	mp (°C)	R_M	log D_{oct}	log k	S (mg/mL)
II	$-CH_3$	301	144–145	−0.52 ± 0.05	1.19 ± 0.09	0.02 ± 0.01	>10[a]
V	$-C_2H_5$	315	182–184	−0.41 ± 0.02	1.51 ± 0.05	0.08 ± 0.02	4.68 ± 0.32
VIII	$-C_3H_7$	329	182–184	−0.21 ± 0.03	1.92 ± 0.06	0.14 ± 0.002	3.96 ± 0.24
XI	$-C_4H_9$	343	162–164	−0.10 ± 0.02	2.33 ± 0.06	0.20 ± 0.003	3.69 ± 0.22
XIV	$-C_5H_{11}$	357	172–174	0.00 ± 0.03	2.75 ± 0.02	0.26 ± 0.003	2.74 ± 0.19
XVII	$-C_6H_{13}$	371	140–142	0.16 ± 0.03	3.29 ± 0.23	0.37 ± 0.002	1.95 ± 0.12
XX	$-C_7H_{15}$	385	121–122	0.35 ± 0.04	3.35 ± 0.02	0.45 ± 0.001	0.13 ± 0.02
XXIII	$-C_8H_{17}$	399	131–133	0.50 ± 0.04	3.50 ± 0.21	0.53 ± 0.003	0.005 ± 0.001
XXVI	$-C_9H_{19}$	413	134–136	0.69 ± 0.05	3.74 ± 0.28	0.62 ± 0.001	—[b]
XXIX	$-C_{10}H_{21}$	427	132–134	0.91 ± 0.04	4.71 ± —[a]	0.68 ± 0.001	—[b]

[a] Only one determination.
[b] Not detectable.
[c] R = substituent in the meta position, MW = molecular weight, mp = melting point, R_M = linear physicochemical descriptor in TLC, log D_{oct} = logarithm of the apparent distribution coefficient at pH 7.4, log k = logarithm of the capacity factor from HPLC (all from 16), S = solubility in 25 mM HEPES-buffered normal saline at pH = 7.4. Mean ± SD, N = 3–6.

μL) was used for HPLC analysis; another part (50 μL) was mixed with 4.5 mL of scintillation cocktail HiSafe (Wallac Scintillation Products, Fisher Chemicals, Loughborough Leics, UK) and used for the determination of ^{14}C-mannitol with liquid scintillation counting (Winspectral 1414 Liquid Scintillation Counter, Wallac, Turku, Finland).

HPLC Assay and Data Analysis

The samples from the permeation experiments via Caco-2 monolayers, as well as from the transdermal experiments, were analyzed by HPLC. The chromatographic system consisted of a membrane degasser, pump (Spectra System P2000), autosampler (Spectra System AS 8000), and UV detector (Spectra Focus), all from Thermo Separation Products (San Jose, California). The column used was Supelcosil™ LC-18-DB 5 μm (Supelco Inc., Bellefonte, Pennsylvania). The mobile phase was a mixture of CH_3OH (HPLC Grade Methanol, Rathburn Chemicals Ltd., Walkerburn, Scotland) and sodium acetate-3-hydrate (Riedel-de-Haen, Switzerland) (6.8 g/L). Ratios of 70:30 (v/v) for the determination of V and VIII; 80:20 (v/v) for the XI, XIV, and XVII; and 90:10 (v/v) for the XX and XXIII were used, respectively. The pH = 6 of all the mobile phases was adjusted with acetic acid (Merck, Darmstadt, Germany). The flow rate of the mobile phase was 0.8 mL/min. The chromatograms were scanned at 246 nm. The injection volume was 10 μL. Data acquisition was carried out by the ChromQuest° Software (ThermoQuest Inc., San Jose, CA).

The apparent permeability coefficients (cm/s) for mannitol and drugs across the cell monolayers were calculated according to equation:

$$P_{app} = \frac{(dO/dt)}{A * 60 * C_0},$$

where *dQ/dt* is the amount of drug or mannitol transported during the interval *dt; A* is the surface area of the insert; 60:1 h = 60 min; C_0 is the initial concentration of mannitol or drug on the donor side. Flux values (*J*) for the drugs and mannitol permeated via the skin (μg/h per cm^2) were calculated by using linear regression analysis of the straight-line portion of the cumulative drug permeated vs. time plots. The permeabilities, K_p (cm/s), of the drugs were calculated by dividing the *J* by the initial donor concentration of the drug.

Determination of the Partition Coefficients and Solubility Parameters

The determination of the partition coefficient (log P_{oct}) by direct measurement using the shake-flask equilibriation method faces problems such as poor reproducibility, length of time for experiment, and needing a reasonable quantity of a compound. These difficulties can be overcome by using reversed phase chromatography (19,20), which has the advantages of the speed of determination, better reproducibility, and the purity of a sample is not a necessary condition. The retention parameter can be log k for HPLC or R_M (R_M = log $1/R_f - 1$) for RP–TLC (21). The apparent partition coefficients (log D_{oct}) of the phenyl carbamic acid esters were determined in n-octanol/phosphate buffer (pH = 7.4). Because high lipophilicity of all the compounds studied was assumed, the amount of organic solvent was small (0.1 mL). The saturation solubilities of the pyrrolidinoethylesters of m-alkoxysubstituted phenylcarbamic acid in the HEPES-buffered saline (pH = 7.4) were measured as three parallel determinations at a room temperature (22°C) after 48 h of agitation. All the drug concentrations were analyzed by HPLC.

RESULTS AND DISCUSSION

The physicochemical properties of the pyrrolidinoethylesters of m-alkoxysubstituted phenylcarbamic acid are listed in Table I. The apparent distribution coefficients of the molecules increase by the lengthening of the side chain. For the compounds V, VIII, XI, and XIV, this increment is regular and has a value of 0.41–0.42. Rekker's model predicts a rise in lipophilicity by a value of 0.52 for each methyl group (22). To characterize or define the lipophilicity of a drug, reversed phase chromatography (RP-TLC and RP-HPLC) has been used (19). As the length of the alkoxychain was increased, the R_M values were increased accordingly (Table I). The same results were obtained from RP-HPLC (log k); the retention times of the compounds studied were increased with the increasing number of carbon atoms in the alkoxychain. Thus, the values of all the lipophilicity parameters (log D_{oct}, R_M, log k) increased with the lengthening of the alkoxychain on the benzene ring (Fig. 1, Table I). Solubility and pH-solubility

Table II. Flux Values and Apparent Permeability Coefficients of the Compounds and Mannitol, TEER Values of the Monolayers Used for the Permeation Experiments, and the Results of the MTT Test

Label		J[a] μg/min)	P^{b}_{app} (cm/s) $\times 10^{-5}$	P^{c}_{app} (cm/s) $\times 10^{-6}$	TEER[d] ($\Omega \times cm^2$)	MTT[e] (%)
V	a-b	0.14 ± 0.006	2.8 ± 0.12	0.16 ± 0.003	452 ± 89	100
	b-a	0.43 ± 0.04	8.7 ± 0.74	0.30 ± 0.02	376 ± 50	
VIII	a-b	0.15 ± 0.01	2.7 ± 0.12	0.17 ± 0.003	389 ± 80	96.6
	b-a	0.27 ± 0.01	6.9 ± 0.25	0.29 ± 0.01	260 ± 82	
XI	a-b	0.15 ± 0.001	2.6 ± 0.01	0.24 ± 0.01	369 ± 106	48.1
	b-a	0.33 ± 0.05	5.9 ± 0.09	0.39 ± 0.002	337 ± 13	
XIV	a-b	0.10 ± 0.006	1.7 ± 0.11	0.69 ± 0.09	486 ± 157	32.7
	b-a	0.41 ± 0.03	7.0 ± 0.048	1.3 ± 0.16	286 ± 27	
XVII	a-b	0.02 ± 0.002	0.33 ± 0.003	3.0 ± 0.43	216 ± 10	4.05
	b-a	0.13 ± 0.02	2.1 ± 0.03	4.5 ± 0.54	122 ± 3	
XX	a-b	0	0	16 ± 5.4	231 ± 19	2.24
	b-a	0.006 ± 0.042	0.66 ± 0.10	10 ± 3.8	123 ± 5	
XXIII	a-b	0	0	—[f]	394 ± 57	—[f]
	b-a	0	0	—[f]	187 ± 21	—[f]

[a] Flux values.
[b] Apparent permeability coefficients of the compounds studied.
[c] Mannitol.
[d] TEER values after 120 min of drug treatment.
[e] Percentage of the activity of cells after drug treatment.
[f] Not determined.
[g] Permeability data were obtained in the apical-to-basolateral (a-b) and basolateral-to-apical (b-a) directions. Mean ± SD (n = 4).

profiles are particularly useful means of identifying compounds likely to have absorption and distribution problems (10). As expected, the solubility of the compounds in HEPES-buffered saline (pH = 7.4) was inversely related to their lipophilicity and decreased with the lengthening of the alkoxychain (Table I).

The initial goal of this study was to perform the permeation experiments of all the 10 compounds from the series (n = 1–10), but for the reasons of inadequate solubility, too low permeability (skin permeation), or cell damage/filter attachment problems (Caco-2), only seven compounds (n = 2–8) were studied experimentally. The measured permeability parameters (flux, J, and the permeability coefficients, P_{app} and K_p) for the Caco-2 and skin experiments are summarized in Tables II and III, respectively.

The Caco-2 permeabilities in both the basolateral-to-apical (b-a) and apical-to-basolateral (a-b) directions against the drug lipophilicity are plotted in Fig. 2. The shape of the curves obtained was similar for the two directions, after an initial plateau a decrease with the lengthening of the alkoxychain (n ≥ 5) was obvious (Fig. 2). In a-b direction the P_{app} values of the compounds V–XVII ranged from 2.8×10^{-5} to 3.3×10^{-6} cm/s. The P_{app} values of XX and XXIII were 0. Thus, the permeabilities of the even more lipophilic XXVI and XXIX were not measured, because the same result was expected. The values of $P_{app}^{b\text{-}a}$ were about 2.5–6-fold higher than the corresponding a-b permeabilities. Most of the commercially successful drugs traverse the cellular barrier by the transcellular pathway, mediated by the passive diffusion of the drug through the apical plasma membrane, across the cell proper, and across the basolateral membrane (1,11). Inhibited (P-glycoprotein-based efflux and CYP 3A4-induced, *e.g.,* metabolism) or active (augmented) transport of drugs in/across the enterocytic membrane is common. To determine whether the phenylcarbamic acid esters are substrates for the P-gp efflux, further studies are required, *e.g.,* with specific inhibitors of P-gp (verapamil, cyclosporin A).

Table III. Transdermal Permeability Coefficients (K_P) for the Molecules Studied

Label	K_P(cm/s) $\times 10^{-8}$	ER
V		
Passive	0.97 ± 0.69	744
Iontophoresis	722 ± 122	
VIII		
Passive	186 ± 64	4.3
Iontophoresis	806 ± 83	
XI		
Passive	219 ± 39	2.8
Iontophoresis	611 ± 178	
XIV		
Passive	214 ± 12	2.5
Iontophoresis	528 ± 172	
XVII		
Passive	214 ± 47	0.79
Iontophoresis	169 ± 39	
XX		
Passive	142 ± 33	1.3
Iontophoresis	186 ± 106	
XXIII		
Passive	0	—
Iontophoresis	0	—

Mean ± SD, (n = 3–6). ER denotes the enhancement ratio (=iontophoretic/passive flux).

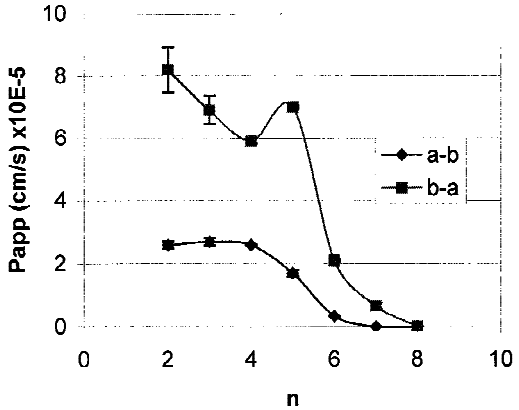

Fig. 2. The apparent permeability coefficients (P_{app}) for the seven esters of phenylcarbamic acid as a function of the length of their alkoxychain. P_{app} was obtained from the permeation studies across Caco-2 cell monolayers in the apical-to-basolateral (a-b) and basolateral-to-apical (b-a) directions. Mean ± SD, N = 3.

The size of a molecule has a major role in its absorption through biological membranes. The paracellular diffusion through the tight junctions as well as the transcellular diffusion is highly dependent on the molecular size of the drug (4,11). Compounds with a molecular weight of about 200–500 Da may diffuse readily through the plasma membrane of enterocytes. Increasing the molecular weight leads to a decrease in membrane diffusion, and the molecular weight cutoff for the Caco-2 cell permeability has been reported to take place at MW = 700 (4) or 600 (23) Da, respectively. According to this finding, the good permeation of the shorter-chained phenylcarbamic acid esters was expected, and the assumption of them traversing the cellular barrier by the transcellular pathway was confirmed.

Two hypotheses may explain the decrease in the permeability of the most lipophilic esters of phenylcarbamic acid. First, it can be due to the fact that the compounds traverse the apical plasma membrane, but not the basolateral one. As a result of their high lipophilicity (log D_{oct} = 3.35–4.71), they may remain in the lipid structures of the membrane. This hypothesis is consistent with the study of Artursson *et al* (11), which reports that the permeability in the intestinal epithelium increases roughly with the lipophilicity of the drug molecule until it reaches a plateau at a log P_{oct} value of about 2. Drugs displaying log P_{oct} values close to 2 are generally predicted to be absorbed completely in humans. For log $P_{oct} > 4$, the permeability starts to decrease, because the very hydrophobic drugs generally have low aqueous solubility and partition at a slower rate from the (lipophilic) cell membranes to the extracellular fluids. A high hydrophobicity and/or low aqueous solubility may complicate transport studies both *in situ* and in cell monolayers. Very hydrophobic drugs may also adsorb to the walls of the transport chambers during the experiment with a large nonspecific loss of the drug as a result. In this study, however, no unspecific adsorption of the phenyl carbamic acid esters could be detected into the plastic (Caco-2 experiments), glass (skin experiments), or HPLC column (drug analysis) materials used.

Second, the very low permeability can be a result of a formation of micelles. Critical micelle concentration values (CMC) for analogical piperidinoethyl esters of alkoxysubstituted phenylcarbamic acids have a magnitude of 10^{-4}–10^{-5} M (24). Higher drug concentrations, which were also used in these experiments (0.25 mM), might lead to the micelle creation, especially by compounds with a longer side chain. These micelles are too big and bulky to traverse the membranes by the transcellular route. On the other hand, 0.25 mM is a rather low drug concentration compared with the saturation solubility of the more hydrophilic esters studied (Table I), and the role of micelle formation is probably minimal with these compounds.

The results of mannitol flux and the values of TEER (Table II) indicate that the integrity of the Caco-2 monolayers after drug treatment is mostly intact. The MTT tests showed that no cell damage was found after the contact with the compounds V and VIII (Table II). After 60 min with XI, half of the cell activity was intact, and XIV caused even more damage, only 30% of the cell activity remained. The more lipophilic compounds, XVII and XX, at the concentration of 0.25 mM, resulted in that only 2–4 % of the cell activity could be detected. Supportive data to these results can be found in an LD_{50} toxicity test of the phenylcarbamic acid esters studied (16). In white mice the *s.c.* LD_{50} was 100–200 mg/kg for the compounds XIV and XVII and 50–100 mg/kg for the XX and XXIII, respectively.

As mentioned before, if the drug displays a log D_{oct} value > 3, its permeability across the Caco-2 monolayer started to decrease rapidly. The reason for this decrease is probably due to the low aqueous solubility of these lipophilic drugs and their slow partitioning from the lipid bilayers of the membrane into the extracellular fluids. At the same time, paracellular permeability of the hydrophilic marker molecule (mannitol) was increasing (Table II), probably because of the partial damage in the cell integrity caused by the most lipophilic model drugs (XVII, XX). MTT test measures the viability of the mitochondria inside the cells (18). Thus, the MTT-test is not a direct measure of the condition/integrity of the cellular barrier. Thus, even if the compound causes a "serious" damage in the function of mitochondria, the barrier properties of the Caco-2 monolayers may remain somewhat undisturbed, possibly, again, because of the fact that the solubilized membrane has a rigid support by the most lipophilic drugs at or near their CMC value.

The permeability coefficients (K_P) for the passive and iontophoretic skin transport experiments are summarized in Table III. Passively, a parabolic relationship of the permeability as a function of drug lipophilicity (chain length) was found with a correlation coefficient of 0.95. The equation for the passive transdermal permeation (K_p passive vs. the number of carbon atoms) was $y = -0.0009\ x^2 + 0.0089 \times - 0.0135$. For the iontophoretic drug permeation (K_p iontophoresis vs. the number of carbon atoms), the equation was $y = 0.0003\ x^3 - 0.0049\ x^2 + 0.0189 \times + 0.006$, with an $R^2 = 0.95$. Similar relationships have been reported in the literature (25,26). A plot of the passive and iontophoretic flux values vs. the number of carbon atoms in the alkoxychain is presented in Fig. 3a. A similar passive permeation study was performed by Le and Lippold (27) with homologous esters of nicotinic acid. The skin permeabilities increased linearly with the extension of the alkyl chain length (log D_{oct} values of 0.85–4.71). In this work, a series of homologous esters with log D_{oct} = 1.51–3.50 gave completely different results, *i.e.,* parabolic dependence between the permeability and lipophilicity, with high perme-

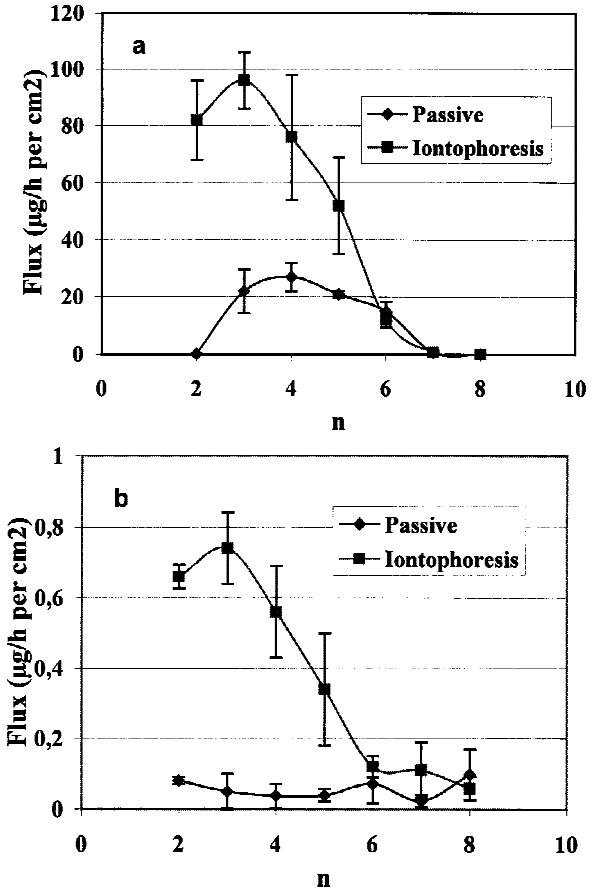

Fig. 3. Plots of the passive and iontophoretic flux values (µg/h per cm^2) through the human skin as a function of the number (n) of carbons in the alkoxychain for (a) the seven esters of phenylcarbamic acid and (b) mannitol. Mean ± SD, N = 3–6.

ability coefficients at lipophilicity values log D_{oct} = 1.92–3.29. The explanation of the different behavior of drugs with similar lipophilicity may lie in the molecular size (MW). According to Camenisch *et al* (5) and Potts and Guy (7), shapes of the lipophilicity vs. permeability plots are size dependent. At low MW the shape is sigmoidal, but the shape takes a bilinear/hyperbolic form for higher MW compounds (5). Obviously, lipophilicity data alone are not sufficient to predict the permeability of drugs. Increase in the lipophilicity by lengthening of the side chain (*e.g.,* incorporation of methyl groups) also means an increase in the MW, which, therefore, does not increase the permeability in all cases. Low permeability by the very lipophilic drugs, *e.g.,* XX in this study, may be caused by the absorption of the drug into the lipids of the skin and/or by a low aqueous solubility (poor partitioning into the receiver phase).

By introducing a low-level electrical current (iontophoresis), the permeability of phenylcarbamic acid esters increased clearly (Table III). A plot of drug flux vs. the number of carbon atoms in the alkoxychain differs clearly from the one obtained for passive diffusion. It has a sigmoidal decreasing character (Fig. 3a). The iontophoretic/passive enhancement ratio (ER) was by far the highest for the most hydrophilic compound, V, studied (Table III). As the length of the alkoxy chain was increased, the enhancement ratio started to decline, and it was nonexistent for the most lipophilic compounds, XVII and XX. This observation is consistent with the model presented by Kontturi and Murtomäki (28): the permeability coefficients of hydrophilic drugs are enhanced significantly by iontophoresis, but only a minor increase in the permeability of lipophilic drugs is observed. Sung *et al* (29) reported that the relative importance of the lipid matrix and aqueous "pores" of the skin under iontophoresis is different. The more hydrophilic (pro)drugs are mainly transported through the aqueous pathway and the more lipophilic drugs primarily through the lipid matrix of *the stratum corneum*.

Thus, even with iontophoretic enhancement, the most lipophilic esters of phenylcarbamic acid could not be transported across the skin (like in the case of Caco-2 permeation). The reason for this cutoff in the skin may lie in the formation of a drug reservoir in the skin during the permeation experiments. The results of mannitol transport (Fig. 3b) support this hypothesis. The lengthening of the alkoxychain (increasing lipophilicity of the drug) not only reduces the iontophoretic permeability of the compounds but also of mannitol. The passive permeation of mannitol was the same with all the compounds (Fig. 3b). This finding indicates that the net negative charge of the skin was neutralized by the iontophoretic delivery of these lipophilic and positively charged phenylcarbamic acid esters. The same behavior (attenuation of electroosmotic solvent flow) has been shown previously in lipophilic, cationic peptides, nafarelin and leuprolide (13,14), and β-blocking agents (15). Thus, close juxtaposition of a positive charge and a lipophilic moiety form an additional obstacle in the iontophoretic permeation of these compounds across the multilayered skin membrane.

It was interesting to see a very good correlation between the K_P of the iontophoretic permeability of the phenylcarbamic acid esters and the effectiveness of surface or infiltration local anesthesia. Correlation coefficients were r^2 = 0.94 and r^2 = 0.92, respectively. The correlation between the K_P of the passive diffusion and the biological activity was very low (r^2 = 0.44 for the surface local anesthesia and r^2 = 0.28 for the infiltration local anesthesia). The values of biological activity were taken from Ref. 16, and they compare the local anesthetic efficacy of the selected phenylcarbamic acid esters with cocaine and procaine.

In conclusion, lipophilicity of the selected esters of phenylcarbamic acid seems to have a very important role in their permeation process, but this parameter alone is inadequate for the prediction of permeability. Very high lipophilicity has a negative influence in both the intestinal and transdermal permeability. Detailed and concentration-dependent cell uptake and skin reservoir formation studies are needed, however, to get a complete mass balance ratios of the absorption of the phenyl carbamic acid esters into the lipidic structures of the Caco-2 and skin membranes. The highest permeabilities were obtained for the compounds with log D_{oct} = 1.5–2.3 (Caco-2 monolayers) or log D_{oct} = 1.9–3.3 (transdermal permeability). Even with iontophoretic enhancement, the most lipophilic derivatives of phenylcarbamic acid could not be transported across the skin, as was the case also with the Caco-2 permeation.

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