Percutaneous Penetration of Nicotinates: In Vivo and In Vitro Measurements

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Abstract □ The relationship between chemical structure and percutaneous absorption has been explored with nicotinic acid and its methyl, ethyl, hexyl, and benzyl esters. Skin penetration has been measured in vitro across hairless mouse skin and in vivo in humans. In vitro, methyl and ethyl nicotinates (when applied in acetone) were delivered into skin such that the stratum corneum barrier was effectively bypassed. The lipophilic esters, on the other hand, were not solubilized in this way and penetrated more slowly. Nicotinic acid penetrated poorly, yielding essentially zero-order skin transport kinetics. Tape-stripping experiments, in which penetration was monitored across skin with no stratum corneum, confirmed these observations. In vivo absorption of the esters was determined from the urinary excretion of total radioactivity following topical administration of ^{14}C-labeled penetrant. Kinetic analysis of the data yielded rate constants, the ratio of which correlated acceptably with the penetrant octanol–water partition coefficient (K). The dependence of the rate constants on K was interpreted in terms of the relative affinity of the substrate for the stratum corneum compared with the viable tissue; the relationship agrees well with a previous evaluation involving structurally unrelated molecules.

Skin penetration is currently attracting much research interest. Percutaneous absorption determines both the efficacy of transdermally delivered local and systemic therapeutic agents and the potential hazard resulting from toxic chemical exposure in the workplace and environment. Understanding skin penetration and its relationship to the chemical properties of the penetrant is essential, therefore, if reliable prediction of the absorption of new or different chemicals is to become possible.

Because there is a transition in skin from cells with a high water content, the dermis and viable epidermis, to the cells of the hydrophobic stratum corneum with a relatively low water content, it is reasonable to expect that the relative lipophilicity:hydrophilicity of the penetrant will be a determinant of skin absorption. A number of in vivo and in vitro studies have demonstrated, as a result, a correlation between percutaneous penetration and a simple oil–water partition coefficient.[1-6] Although quantitative extrapolation from these observations cannot be justified at this time, it is clear that an effective skin penetrant requires both lipophilic and hydrophilic properties.

To identify experimentally the relationships between chemical structure and skin penetration, data of greatest relevance will be provided by conducting in vivo experiments in humans. However, this approach is not always possible since considerable time and resources are required and many chemicals of interest are toxic and too hazardous to test in humans. Alternative methodologies must then be considered; for example, the use of an in vivo animal model[7,8] or the in vitro assessment of transport across excised (human or animal) skin tissue. In the latter category, hairless mouse skin is a frequently used model. It is convenient, easy to obtain, and appears to provide relatively consistent barrier properties. Although it differs histologically from human skin, it has proven to be a useful skin membrane for elucidating mechanistic aspects of the absorption process.[9]

The experiments reported here attempt to consider the skin penetration–chemical structure relationship for a series of nicotinate derivatives. Although the penetration of these substances has been studied pharmacodynamically via their cutaneous vasodilatory effect,[10] absorption has not been measured quantitatively. Experimentally, transdermal transport has been followed both in vivo in humans and in vitro across the excised skin of hairless mice. The overall objective was to explore, in two complementary experimental systems, the functional dependence of absorption on penetrant physicochemical characteristics.

Experimental Section

In Vitro Experiments—In vitro experiments were performed using continuously perfused glass diffusion cells (Laboratory Glass Apparatus Inc., Berkeley, CA).[11] The skin membrane was full-thickness tissue excised from the back of hairless mice (Skin Cancer Hospital, Philadelphia, PA; SKH:HR-1), aged 5–15 weeks. Animals were sacrificed by CO_2 euthanasia followed by bilateral thoracotomy, and the excised skin was used within 48 h. The perfusate was phosphate-buffered saline, pH 7.4, and the flow rate from a peristaltic pump (Manostat, New York, NY) was set at 10 mL/h, i.e., one cell volume per hour. The receptor phase was stirred throughout the experiments and was collected serially in separate fractions using a programmed collector (Gilson FC 220, Middleton, WI) and a peristaltic pump. The area of the skin exposed to chemical was 3.14 cm^2. The dose of penetrant was 4–5 $\mu g/cm^2$ (12–16 μg/cell) dissolved in 40 μL of acetone. Carbon-14 labeled compounds were applied and penetration was measured by counting the radioactivity in each sample. At the end of the experiment the skin surface was washed with 3 × 2.5 mL of distilled water and the skin was then digested in tissue solubilizer (Soluene 350, Packard Instruments, Downers Grove, IL). All radioactivity was determined by liquid scintillation counting.

Total Absorption Determination—For all compounds studied (nicotinic acid, and its methyl, ethyl, hexyl, and benzyl esters), the 48-h penetration time course was first determined. During the first 2–3 h postapplication, the receptor phase was collected in 10–20 min intervals. Thereafter, the perfusate was collected every hour until the end of the run. The experiments lasted 24–48 h. For each compound, four pieces of hairless mouse skin were used. Based on total recovered radioactivity and radioactivity per fraction, the percent dose per hour reaching the fraction collector was calculated.

Stripped Skin Experiments—Since stratum corneum is often the major barrier to the penetration of chemicals through skin, the absorption kinetics of three penetrants (nicotinic acid, methyl nicotinate, benzyl nicotinate) were studied after removal of the stratum corneum.[9] For each penetrant, two pieces of skin were "stripped" with tape (Scotch Magic Transparent Tape, 3M, St. Paul, MN). The stratum corneum was considered to be completely removed when the skin appeared shiny and moist ("glistened"). This could be achieved after as few as seven strippings. The stripped skin was mounted in the diffusion cell, and penetration was followed as described above.

In Vivo Experiments—The in vivo penetration of methyl, ethyl, hexyl, and benzyl nicotinates was studied using conventional methodology.[12] Each compound was administered topically to four subjects. The application site was the forearm and the dose was 4 $\mu g/cm^2$

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in an acetone vehicle. The amount of radioactivity applied was 2–5 μCi. Thus, the area of application was determined by the specific activity of the compounds. After application of the test compound, urine was collected for 5 d according to the following schedule: 0–4 h, 4–8 h, 8–12 h, 12–24 h, 24–36 h, 36–48 h, day 3, day 4, and day 5. The application site was covered with a piece of nonocclusive gauze during the first 24 h, and the subjects did not wash the area during this time. The volume of the urine was determined gravimetrically for each time period, and duplicate 5-mL samples were assayed for radioactivity. [^{14}C]Toluene (internal standard) was added to a third 5-mL sample and was counted to determine quenching. The percent "dose" (as total radioactivity) excreted was determined for each time interval.

Partition Coefficient Determinations—Partition coefficients of methyl and ethyl nicotinates were obtained from the literature.[13,14] The partition coefficients for nicotinic acid, hexyl nicotinate, and benzyl nicotinate were determined classically by allowing a known concentration of compound in aqueous solution to equilibrate with a known volume of octanol at 37°C for 3 d. Final concentrations of nicotinate in the two phases were determined spectrophotometrically. For hexyl nicotinate, the solute was dissolved in octanol (due to its very low water solubility), and 1 mL of this solution was then equilibrated with 10 mL of water. For nicotinic acid, K was determined at pH 3.1 and 4.5. Carbon-14 labeled nicotinic acid, which had been purified by thin-layer chromatography (TLC), was dissolved in a buffer at the appropriate pH and was then equilibrated with octanol as described above. At the end of the partitioning, the amount of carbon-14 in both the aqueous and organic phases was counted. The counting efficiency in the two layers was determined using [^{14}C]toluene as the internal standard.

Chemicals—Carbon-14 labeled nicotinic acid (56 mCi/mmol) and benzyl nicotinate (3.7 mCi/mmol) were purchased commercially (New England Nuclear, Boston, MA). The label was incorporated at the carboxy carbon atom. Radiolabeled methyl, ethyl, and hexyl esters were synthesized from [^{14}C]nicotinic acid.

Methyl Nicotinate—Methylation of [^{14}C]nicotinic acid was achieved using diazomethane as the esterification agent.[15] The ester was purified by TLC. Yields in excess of 60% were obtained.

Ethyl Nicotinate—Carbon-14 nicotinic acid (16 μmol) was mixed with 1.95 mL of ethanol and 0.26 mL of sulfuric acid. The mixture was refluxed for 4 h, cooled, and then neutralized with potassium carbonate. The ester was extracted with ether and the purity checked using TLC (yield = 49%).

Hexyl Nicotinate—Carbon-14 labeled hexyl nicotinate was synthesized by mixing 10 μmol of carbon-14 nicotinic acid with 0.1 mmol of hexanol, 0.12 mmol of 2-chloro-1-methylpyridinium iodide, and 0.12 mmol of triethylamine in a dichloromethane solution.[16] The mixture was stirred overnight at room temperature under argon. The radiolabeled hexyl nicotinate was then purified by TLC (yield = 75%).

Results

In Vitro Experiments—Figure 1 shows the complete in vitro absorption profile for the five nicotinate derivatives studied. Each curve is the mean of four separate determinations. On average, the standard deviation about each data point was approximately 10%.

Figure 2 illustrates the penetration profile observed for nicotinic acid, methyl nicotinate and benzyl nicotinate across stripped hairless mouse skin. In this case, each curve is the mean of two virtually superimposable, separate, determinations for each compound.

In Vivo Experiments—In vivo percutaneous absorption results for the nicotinate esters are summarized in Table I. The data have been corrected for incomplete urinary excretion using a standard approach.[12]

Partition coefficients—Octanol-water partition coefficients are given in Table II.

Data Analysis

In Vitro Experiments—To interpret the total penetration profiles for the five penetrants (Fig. 1), the model postulated

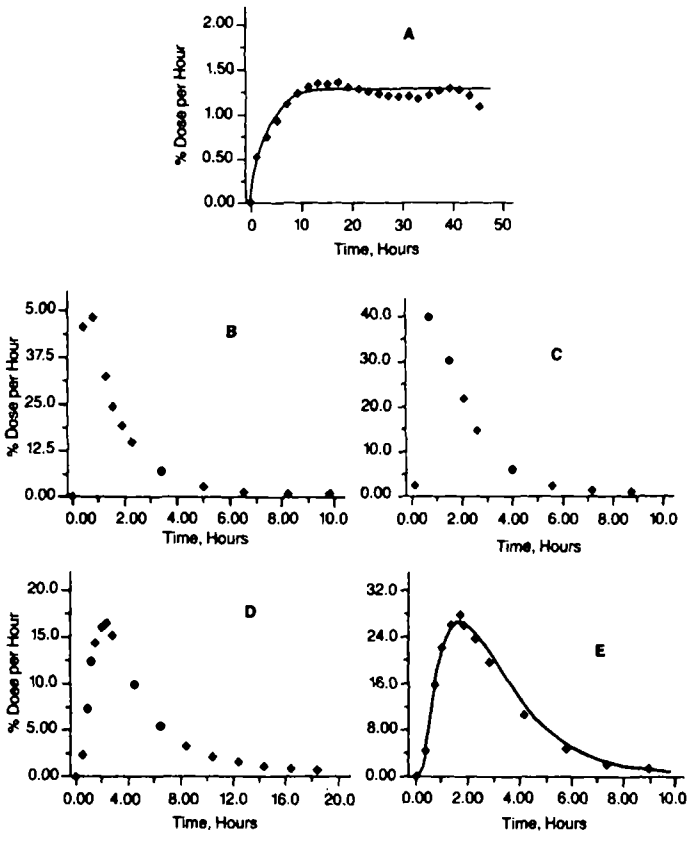


Figure 1—*In vitro absorption profiles across full-thickness, hairless mouse skin for the five compounds studied:* (A) *nicotinic acid;* (B) *methyl nicotinate;* (C) *ethyl nicotinate;* (D) *hexyl nicotinate;* (E) *benzyl nicotinate. The curves shown are the means of four separate determinations. The variation about each data point is, on the average,* ≤ ± *10%. The smooth curves in* A *and* E *are the 'best' fits to the data using eqs. 2 and 1, respectively (parameter estimates are given in Table III).*

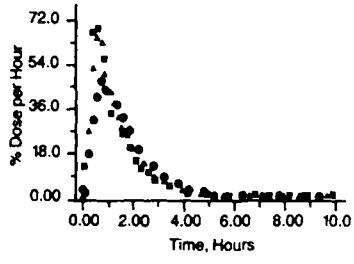


Figure 2—*In vitro penetration profiles of three compounds through stripped, hairless mouse skin. Key:* (■) *nicotinic acid;* (▲) *methyl nicotinate;* (●) *benzyl nicotinate. The curves shown are the averages of two essentially superimposable, separate, determinations.*

by Mathias et al.[11] (Scheme I) was used. This model adequately described the absorption of benzoic acid and paraquat through hairless mouse skin following topical delivery in a volatile solvent vehicle. The model allows for the possibility that, on application, a fraction (X_{20}) of the total penetrant dose ($X_{10} + X_{20}$) is immediately delivered into the skin. Calculations of the rate parameters of the model and the values of X_{10} and X_{20} suggested by the data are achieved by extended least-squares regression.[17] The process is facilitated by the fact that k_{34} can be fixed since it represents the clearance of penetrant from the receptor phase by the perfusion system. Hence, k_{34} is equivalent to the flow rate (10 mL/h) divided by the receptor chamber volume (10 mL), i.e.,

Table I—In Vivo Skin Absorption of ^{14}C-Labeled Nicotinates[a]

Time at midpoint of collection interval, h	Rate of excretion, % dose/h			
	Methyl	Ethyl	Hexyl	Benzyl
2	0.104	0.100	0.117	0.123
6	0.462	0.181	0.132	0.181
10	0.308	0.142	0.166	0.434
18	0.145	0.094	0.258	0.293
30	0.127	0.106	0.325	0.286
42	0.101	0.061	0.209	0.258
60	0.097	0.058	0.179	0.257
84	0.076	0.056	0.126	0.137
108	0.094	0.044	0.116	0.214

[a] Mean (n = 6) urinary excretion of ^{14}C-labeled nicotinates as a function of time postadministration. The data have been corrected for incomplete urinary excretion using a standard approach (ref 12).

Table II—Octanol–Water Partition Coefficients (K) of Nicotinates

Penetrant	K (%SD)	
Nicotinic Acid	0.15	(0.7)[a]
Methyl Nicotinate	11	(—)[b]
Ethyl Nicotinate	30	(—)[c]
Hexyl Nicotinate	3910	(8)
Benzyl Nicotinate	254	(16)

[a] Value determined at pH 4.5; K = 0.22 (±8%) at pH 3. [b] Literature value from ref 13. [c] Literature value from ref 14.

$k_{34} = 1\ h^{-1}$. Regression analysis of the data involves determination of the best fit of the model to the experimental results. For the four nicotinate esters, input from the skin is first-order and the data are fit, therefore, to eq. 1:

$$dA_4/dt = k_{12}k_{23}k_{34}X_{10}\{\exp(-k_{12}t)/[(k_{23} - k_{12})(k_{34} - k_{12})] + \exp(-k_{23}t)/[(k_{12} - k_{23})(k_{34} - k_{23})] + \exp(-k_{34}t)/[(k_{12} - k_{34})(k_{23} - k_{34})]\} + k_{23}k_{34}X_{20} \times \{[\exp(-k_{23}t) - \exp(-k_{34}t)]/(k_{23} - k_{34})\} \quad (1)$$

where dA_4/dt is the rate of appearance of penetrant in the fraction collector vials. For nicotinic acid, which is absorbed much less efficiently and results in essentially steady-state behavior, input was modeled with a zero-order process (k^0) and the data were interpreted with eq. 2:

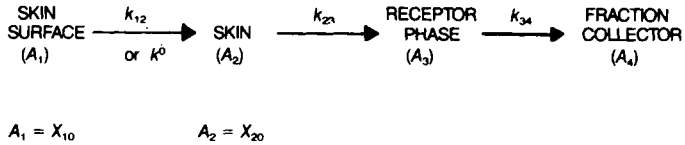
$$dA_4/dt = k^0k_{23}k_{34}\{1/k_{23}k_{34} - \exp(-k_{23}t)/[k_{23}(k_{34} - k_{23})] - \exp(-k_{34}t)/[k_{34}(k_{23} - k_{34})]\} + k_{23}k_{34}X_{20} \times \{[\exp(-k_{23}t) - \exp(-k_{34}t)]/(k_{23} - k_{34})\} \quad (2)$$

The regression parameters obtained in this way for the five penetrants are collected in Table III. The smooth curves in Figs. 1E and 1A illustrate the fits of eqs. 1 and 2 to the data for benzyl nicotinate and nicotinic acid, respectively.

In Vivo Experiments—The in vivo absorption data were analyzed with the biophysical kinetic model of Guy et al.[18] (Scheme II). The k_1 rate constant describes diffusion of chemical through the stratum corneum and can be estimated[19] via the Stokes–Einstein equation from the molecular weight of the penetrant (M_R^u) and the known value of k_1 for benzoic acid (k_1^{BA}) having molecular weight M_R^{BA}:

$$k_1 = k_1^{BA}\,[M_R^{BA}/M_R^u]^{1/3} \quad (3)$$

The k_2 rate constant relates to diffusion of the penetrant through the viable epidermis. As for k_1, k_2 can be estimated from known values of k_2^{BA} and M_R^{BA}, correcting for molecular size, with an expression analogous to eq. 3.[19] The k_3 rate constant describes the affinity of the penetrant for the stratum corneum compared with the viable epidermis. The ratio k_3:k_2 may be regarded as an "effective" partition coefficient. The greater the value of k_3, the more the drug is held back in (and the more it interacts with) the stratum corneum. The k_4 rate constant is the elimination rate constant of the penetrant as determined, in most pharmacokinetic studies, following intravenous administration of the compound. For some penetrants, more complicated removal kinetics are observed and must be incorporated.



Scheme I

Scheme I—Simple kinetic model (ref 11) used to analyze the in vitro penetration results.

Four differential equations describe the model depicted in Scheme II. These expressions describe the rates of change of penetrant concentration on and within the skin, in the blood, and in the urine. They may be solved for dA_u/dt, the rate of appearance (dose fraction per unit time) of penetrant in the urine, as a function of time, t:

$$dA_u/dt = Fk_1k_2k_4\{\exp(-k_1t)/[(k_1 - \alpha)(k_1 - \beta)] + \exp(-\alpha t)/[(\alpha - k_1)(\alpha - \beta)] + \exp(-\beta t)/[(\beta - k_1)(\beta - \alpha)]\} \quad (4)$$

where F is the fraction of the total amount of penetrant in contact with the skin at $t = 0$ which eventually penetrates, and α and β are the roots of the quadratic: $s^2 + (k_2 + k_3 + k_4)s + k_2k_4 = 0$.

In analyzing the in vivo results for the nicotinate esters, the k_1 and k_2 parameters were determined as described above, and the k_4 and F parameters were measured experimentally. Then, k_3 was obtained for each penetrant by finding the best fit of eq. 4 to the in vivo results. The rate constants are collected in Table IV and Fig. 3 compares the experimental data with the theoretical simulations.

Discussion

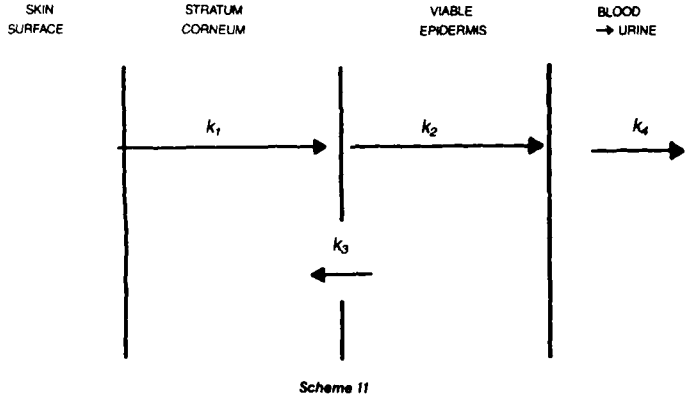
The data analysis of the in vitro results for the nicotinate esters identifies two types of behavior, one associated with the more polar derivatives (methyl, ethyl) and the second with the lipophilic molecules (benzyl, hexyl). For methyl and ethyl nicotinates, it appears that 80–90% of the applied dose is made immediately available within the skin. The remainder, which is left on the surface, is absorbed much more slowly. Benzyl and hexyl nicotinates, on the other hand, do not appear to be delivered directly into the skin to any appreciable extent. The difference in their absorption profiles is accounted for by the difference in estimated k_{12} values for these penetrants. Skin transport is implicated, therefore, as the rate-limiting absorption step, a conclusion supported by the high (and very similar) k_{23} kinetics for these esters.

In vitro penetration of nicotinic acid implies a substantial period of steady-state transport. The behavior is suitably modeled by assuming a zero-order input function (see Table III and Scheme I). We may consider, in this case, that the stratum corneum is acting as a rate-limiting membrane for transdermal delivery of this compound. The stripping experiments support this conclusion and show that the three molecules tested, which have considerably different physicochemical properties, all penetrate with equal efficiency when the stratum corneum is removed. The enhanced delivery of the methyl ester through intact skin is also confirmed by the

Table III—Kinetic Parameters using the Model Shown in Scheme I[a]

Parameter	Nicotinic Acid	Methyl Nicotinate	Ethyl Nicotinate	Hexyl Nicotinate	Benzyl Nicotinate
k_{12}, h^{-1}[b]	—	0.04 (±310%)	0.04 (±365%)	0.26 (± 1.3%)	0.57 (± 4.1%)
k^o, % dose/h[c]	1.27 (± 1.3%)	—	—	—	—
k_{23}, h^{-1}[d]	0.28 (±13%)	2.20 (± 14%)	1.24 (± 12%)	3.11 (±25%)	3.84 (±20%)
X_{10}[e]	0.99 (± 0.4%)	0.18 (± 54%)	0.11 (± 29%)	1.00 (± 1.3%)	1.00 (± 1.7%)
X_{20}[e]	0.01	0.82	0.89	0.00	0.00

[a] Parameters (± %SD) determined by least-squares analysis of in vitro absorption results using model postulated in ref. 11. [b] Kinetics of transport between skin surface and skin (Scheme I). [c] Assuming a zero-order process (Scheme I). [d] Kinetics of transport between skin and receptor phase (Scheme I). [e] Fraction of the total penetrant dose ($X_{10} + X_{20}$).



Scheme II—Biophysical model (refs 18 and 19) of percutaneous absorption employed to analyze the in vivo penetration results.

stripping results: there is little difference in the penetration of methyl nicotinate whether through tape-stripped or undamaged tissue.

The observation that delivery of methyl and ethyl nicotinates in acetone can effectively by-pass the stratum corneum barrier is remarkable. As will be shown below, this effect is not manifested in vivo in humans. The action is specific to the methyl and ethyl esters; the data for nicotinic acid and hexyl and benzyl nicotinates do not show similar behavior. Explanation of these results is not immediately obvious. If acetone causes structural damage to the stratum corneum (cf., stripping), then one might expect all materials to be similarly affected. The uniqueness with respect to the short-chain esters implies a facilitation mechanism which is dependent on penetrant physicochemical properties. An argument on the basis of lipid solubility provides a possible interpretation. First, acetone is able to promote the ready solubilization of methyl and ethyl nicotinates into the stratum corneum. Their partitioning properties imply that they will have acceptable solubility in the horny layer; the acetone speeds the process of dissolution considerably. Second, nicotinic acid, on the other hand, is relatively lipid insoluble and distributes poorly into the stratum corneum, even in the presence of acetone. Finally, the hexyl and benzyl esters are freely soluble in the outermost layer of skin and acetone alters this situation very little. The ultimate appearance of these compounds in the receptor phase may be controlled to a significant extent by their rate of transport out of the stratum corneum into the more aqueous viable tissue. The volatile solvent cannot affect this process and no apparent perturbation is seen in the results.

The absence of perceived parallel behavior in vivo in humans is probably a reflection of the differences between hairless mouse and human stratum corneum. The murine barrier is thinner than that of humans. A greater volume of acetone (or prolonged contact period) is probably necessary for absorption changes to be manifest through human skin.

Table IV—In Vivo Percutaneous Absorption of Nicotinates[a]

Parameter	Methyl Nicotinate	Ethyl Nicotinate	Hexyl Nicotinate	Benzyl Nicotinate
F^b	0.085	0.085	0.21	0.28
k_1, h^{-1}[c]	0.18	0.17	0.15	0.15
k_2, h^{-1}[d]	2.79	2.70	2.43	2.41
k_3, h^{-1}[e]	5.0	15	30	30
k_4, h^{-1}[f]	0.17	0.17	0.17	0.17
$k_3:k_2$[g]	1.8	5.6	12.4	12.5

[a] Parameters determined for application of the biophysical model shown in Scheme II (ref 18). [b] The fraction of the total amount of penetrant in contact with the skin at $t = 0$ which eventually penetrates the skin. [c] Rate constant describing the diffusion of the penetrant through the stratum corneum. [d] Rate constant describing the diffusion of the penetrant through the viable epidermis. [e] Rate constant describing the affinity of the penetrant for the stratum corneum compared with the viable epidermis. [f] Elimination rate constant of the penetrant. [g] "Effective partition coefficient".

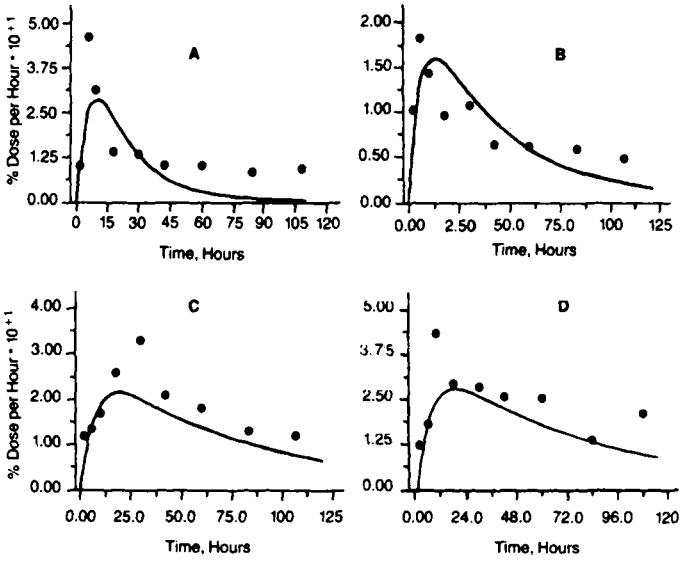


Figure 3—*In vivo percutaneous absorption of four nicotinate esters. Comparison between the experimental results and the theoretical simulations of the model in Scheme II using the rate constants in Table IV:* (A) *methyl nicotinate;* (B) *ethyl nicotinate;* (C) *hexyl nicotinate;* (D) *benzyl nicotinate.*

The in vivo results in Table I and Fig. 3 show that the pharmacokinetic model (Scheme II) can adequately analyze human skin penetration data. For the approach to be useful, the estimated parameter (k_3) must in some way be predictable from the molecular properties of the penetrant. The significance of k_3 lies in the suggestion that the ratio $k_3:k_2$ reflects an "effective partition coefficient" of the penetrant between the stratum corneum and the viable epidermal tissue. It should be possible, therefore, to correlate this ratio

with an oil–water distribution coefficient (K), and then to use the derived relationship and K to predict k_3:k_2. Such a procedure has been reported[19] using the octanol–water partition coefficient as the model distribution parameter. Linear regression of the estimated k_3:k_2 values for the four esters against their respective octanol–water partition coefficients (K; Table II) gives: $\log (k_3/k_2) = 0.30 (\log K) + 0.16$, with $r^2 = 0.71$. This result may be compared with a previous correlation involving nine chemically unrelated penetrants for which the slope was 0.36 and the intercept 0.10.[19] The agreement is good and implies that the kinetic approach may prove to have predictive value for human percutaneous absorption.

Comparison between the in vivo results reported in this paper and previous human studies using nicotinic acid esters is not possible. The earlier work measured, either visually[10,21–26] or using laser Doppler velocimetry,[27–30] the onset and duration of erythema following topical application of nicotinate in aqueous solutions. These effects occurred within minutes as opposed to the significantly prolonged time scale of the current quantitative observations. Correlation between the cutaneous pharmacodynamic reaction and the systemic kinetic behavior is not a trivial task and is beyond the scope of this report.

In conclusion, the experimental findings presented contribute to the database addressing the relationship between chemical structure and properties and percutaneous absorption. It has been demonstrated in vivo, in humans, that the series of nicotinate esters behave in a fashion consistent with that of a set of other, structurally unrelated, compounds.[19] In vitro, using hairless mouse skin as the model membrane, the 'simple' delivery system of a small volume of acetone has been shown to exert effects which (probably) depend on the lipid solubility of the penetrant.

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