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Research Paper

Estimation of Transdermal Permeation Parameters in Non-stationary Diffusion Experiments. Application to Pre-treatment Studies with Terpenes

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Purpose. To estimate the applicability of transdermal drug permeation parameters in a finite-dose model for skin pre-treated with terpenes and to evaluate the enhancing effect of some terpene formulations on alprazolam permeation.

Methods. *In vitro* enhancement of alprazolam human skin permeation was investigated using a pre-treatment with different terpene solutions. Vertical diffusion, Franz-type cells were used. Intrinsic drug permeation was also investigated. Transdermal permeation parameters were estimated from the permeation tabulates using different theoretical approaches for their calculation. Two groups of permeation parameters were calculated: modelistic (diffusion of a finite-dose of drug model) and parameters non-dependent of a diffusional model.

Results. In control experiments, all approaches of data treatment satisfactorily described the experimental permeation profiles. When skin pre-treatment was investigated, the fitting of a mathematical sigmoid function was much better than the diffusional approach. Pre-treatment of the skin with Limonene dissolved in ethanol / propylene glycol and Menthol dissolved in propylene glycol increased 15 and 10 times respectively the permeation parameters of alprazolam.

Conclusions. Using enhancers that are rapidly cleared from the skin, skin permeability does not remain constant during the permeation experiment and therefore it is not possible to calculate parameters that are usually true coefficients or definite values. In this case, non-modelistic parameters can be used to estimate an enhancing effect.

KEY WORDS: permeation rate; transdermal flux; finite-dose diffusion model; non-modelistic parameters; permeation enhancers.

INTRODUCTION

The suitability of a drug for transdermal administration depends on its particular biopharmaceutic, pharmacokinetic, and pharmacological properties. Intrinsic transdermal flux does not usually allow systemic plasma levels of therapeutic significance to be reached. Thus, chemical substances can be used to improve drug diffusion or partition across the epidermic stratum corneum.

The skin permeation enhancing action of terpene substances has been progressively demonstrated for specific drugs. In particular, enhancing activity has been reported for hydrophilic or hydrophobic drugs (1,2). Some authors (3) have postulated that these substances increase intermembrane drug diffusion and that their activity depends on their own diffusion across the membrane.

A useful model for experimental testing of drug permeation enhancers is based on the pretreatment of the skin with enhancer formulation candidates, followed by a permeation study of the drug over the same skin specimen. In this case, drug permeation is run for a series of substances, obtaining results that can be analyzed comparatively. This method enables to use the same thermodynamic activity of the drug in all the formulations. It represents an advantage for comparing activities of different enhancers in drug permeation. Robust conclusions can thus be extracted and it has been used by different authors for formulation optimization (4,5). Whereas in the past, transdermal permeation studies usually used biophysically explicative models, research has progressively evolved toward the use of more practical approaches to formulation optimization.

Drug diffusion from an aqueous solution accross the skin can be considered as a passive process and its kinetic evolution can be described using Fick's classic diffusion equation. In this process, following a drug incorporation stage, a stationary stage is reached and thus a linear relationship between drug permeation and time can be observed (infinite-dose model). If drug concentration in donor compartment decreases significantly with time, a kinetic modification must be included in the model (6). This finite-dose diffusion model is useful for diffusion studies whenever solutions of drugs with a low solubility/permeability balance are studied or when drug at low concentration in respect of its solubility is used as donor formulation. For this reason, it is commonly used for comparing enhancing activities. The present study reports a set of results that were found not to satisfy this generally useful approach, and some mechanistic explanations for this fact are proposed.

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The transdermal permeation of a benzodiazepine, Alprazolam, was assayed and, in order to enhance its skin permeation rate, different monoterpene compounds were tested. Abdominal human skin was used as a diffusion membrane, and a pretreatment model was considered for evaluation.

The main aim of this work was to calculate different drug permeation parameters from a set of experimental results and to evaluate the advantages or limitations of each mathematical approach. At the same time, the activity of different terpene formulations on the transdermal permeation of alprazolam in an experimental study of *in vitro* permeation across human skin pretreated with terpenes was investigated.

MATERIALS AND METHODS

Materials

Chemicals

Deionized water, methanol, and propylene glycol were of analytical grade. 1,8-cineol, (±)-menthol, (+)-limonene, (–)-menthone, and 7-oxabicyclo[2.2.1]heptane were purchased from Sigma Chemical Co. (Alcobendas, Madrid, Spain). Alprazolam was kindly provided by Pharmacia-Upjohn (Madrid, Spain), and diazepam (used as internal standard) was purchased from Acofarma (Barcelona, Spain.).

Skin Preparation

Abdominal human skin (from females aged 26–48 years) was obtained following plastic surgery. Fat excess was removed with a scalpel and the sample was frozen at –20°C. Skin sections of 400 μm thickness were then cut using an electric dermatome (Aesculap AG, Tuttlingen, Germany) and pieces of about 8 cm^2 were obtained and stored. These pieces were passively defrosted overnight at 4°C for diffusion study.

Estimation of Alprazolam Solubility Under the Experimental Conditions

Twenty milligrams of alprazolam was dispersed in 5 ml buffer phosphates, pH 7.4 at 32°C, avoiding evaporation, and shaken continuously for 20 h. For dissolved alprazolam quantitation, 1.5 ml aliquots were taken and centrifuged in 4-ml conical tubes (4000 rpm/10 min) at 35°C. Fifty microliters of the supernatant was taken and diluted for HPLC quantitation. This procedure was performed in quadruplicate on three different days. The mean of twelve results was taken as the alprazolam saturation concentration under the specific working conditions.

Permeation Experiments

Experiments were performed *in vitro* using vertical diffusion cells mounted in a six-cell drive system as part of a Microette autosampling apparatus (Hanson Research Corp., Chatsworth, CA, USA). The cells had a cross-sectional area of 2.54 cm^2 and the receptor compartment had a mean volume of 12 ml. The epidermic surface was maintained at 32°C and the receptor compartment filled with phosphate buffer solution (pH 7.4), as commonly used (7,8), assuring sink conditions.

Five monoterpenes with different lipophilicities were tested. All terpenes were dissolved in propylene glycol (PG) at ratios 20:80 (w/w) and d-limonene required ethanol for complete solubilization. It was finally dissolved at 20:16:64 (limonene/PG/ethanol). The formulations that were used are summarized in Table I.

In each permeation experiment, six skin pieces from the same donor were used. Five of the six cells were used for testing each enhancer preparation, while in the sixth no enhancer was applied. Results from this last cell enabled to obtain the baseline permeation profile of alprazolam for this skin donor. Each terpene formulation was tested with skin pieces from two or three different donors.

Skin Pretreatment and Drug Permeation

Skin pieces were mounted in the diffusion cells. Three hundred μl of one of the enhancer solutions listed in Table I or 500 μl phosphate buffer (pH 7.4) were then placed in the donor compartment of each cell for 6 h. After 6 h of pretreatment, the enhancer solution was drawn by aspirating the residual content of the donor compartment; the skin surface was then rinsed with propylene glycol and increasing dilutions of propylene glycol:buffer pH 7.4.

Immediately after, 1.5 ml of an alprazolam solution (70 μg/ml in phosphate buffer, pH 7.4) was placed in the donor compartment in order to begin the drug permeation phase. Receptor solution samples were taken at the following times: 30 min, and at 2, 4, 6, and 8 h, followed by fixed 4-h intervals until the final time of 24 h. Baseline drug permeation was obtained from the corresponding skin specimens used on each pretreated profile.

Analytical Quantitation

Alprazolam was quantitated by means of an HPLC-UV method. An internal standard (diazepam) was used for assuring the possibility of reanalysis of the samples.

A C18 reversed phase radial compression column (NovaPak C18, 5 mm × 10 mm, 4 μm, Waters, Milford, USA) was used. The mobile phase was a binary combination of methanol:water (60:40), eluting with a flow of 2 ml/min. Samples were quantitated at 222 nm. Calibration was performed between 0.03 and 10 μg/ml and accuracy and precision were calculated. Concentrations below 0.03 μg/ml were considered as non-quantifiable.

Alprazolam permeated amounts were calculated based on alprazolam concentrations on the samples respective cell volumes. Thus, the corresponding tabulated values of cumulative permeated amounts/time were obtained.

Table I. Solutions Used on Skin Pretreatment

Permeation enhancer	Solvent	Enhancer:solvent ratio	Abbreviation
1,8-Cineol	PG[a]	20:80	CIN
(±)-Menthol	PG	20:80	MNT
(+)-Limonene	PG/ethanol	20:16:64	LIM
(–)-Menthone	PG	20:80	MNN
7-Oxabicyclo [2.2.1] heptane	PG	20:80	BCL

[a] Propylene glycol.

Calculation of Permeation Parameters

Experimental results were analyzed by means of two calculation approaches: 1) parameters representative of the finite-dose diffusion model; 2) parameters independent of the diffusion model.

For each calculation option, results were analyzed and studied for this specific case of terpene pretreatment of the diffusion membrane.

Permeation Parameters Derived from the Mathematical Fitting of the Representative Equation of Finite-Dose Diffusion

Equation 1 (6) describes the evolution of the Laplace transform for the accumulated amount of solute which penetrates a homogeneous membrane into the receptor compartment under sink conditions from a donor solution at decreasing concentration, as assumed in the finite-dose diffusion model.

$$\overline{Qr} = \frac{A\,P_1\,Q_0}{s\left[V_d\sqrt{\frac{s}{P_2}}\sinh\sqrt{\frac{s}{P_2}} + P_1\,A\cosh\sqrt{\frac{s}{P_2}}\right]} \quad (1)$$

where $\bar{Q}_r$ is the transformed variable of the permeated amount of drug, s is the Laplace variable, V_d is the donor solution volume, Q_0 is the initial drug amount in the donor compartment, A is the effective permeation surface, and P_1 and P_2 are representative parameters of the membrane/donor solution drug partition coefficient (K) and drug diffusion coefficient (D), respectively, as defined elsewhere (9,10):

$$P_1 = K \cdot L \quad (2)$$

$$P_2 = \frac{D}{L^2} \quad (3)$$

where L is the mean membrane thickness

Permeation parameters P_1 and P_2 were calculated by means of fitting the adequate solution from Fick's second law of diffusion (Eq. 1) to the experimental data by means of the Laplace computer program (Micromath Scientific Software). This program uses the Weeks numerical inversion algorithm (11) for Laplace transforms, followed by the optimization, through minimization, of the residual sum of squares in order to achieve the best possible solution.

Specifically, the following permeation parameters were calculated:

- Permeability coefficient (K_p). The permeability coefficient (K_p) was calculated as the product of parameters P_1 and P_2:

$$K_p = P_1 \cdot P_2 \quad (4)$$

  The numerical value was expressed multiplied by a factor of 10^6.

- Maximum flux (J_{max}) and time to attain this value ($t\,J_{max}$). In this finite-dose model, the transdermal flux (J), or permeation rate, is not a constant value. It rises to reach a maximum (J_{max}) before falling down progressively. Thus, the solute permeation rate through the membrane:

$$J = \frac{dQ}{dt} \quad (5)$$

  was calculated for this model by applying Laplace transforms, giving:

$$\bar{J} = Q \cdot s \quad (6)$$

  Equation 6 was used for the calculation of the predicted values of flux (J) as a function of the experimental time.

- Mean transit time of the drug through the skin (MTT). Based on this finite-dose model, MTT values were calculated using the representative parameters of diffusion (P_2) and partition (P_1) by means of Eqs. 4 and 7:

$$MTT = \frac{1}{2P_2} + \frac{V_d}{K_p\,A} \quad (7)$$

  where V_d is the donor volume value and A the effective permeation surface.

- Theoretical quantity of permeated drug predicted at the end of the experiment (Q_{24}cal) was estimated by means of Eq. 1.

Parameters Independent of the Diffusion Model

The following permeation parameters, independent of the diffusion model, were used:

- Permeated quantity of drug at 24 h, as an experimental value (Q_{24}obs).
- Area under the permeated amounts/time curve as an indication of the extent of drug passing through the skin (AUC_0^t).

For AUC_0^t calculation, a continuous plot of the evolution of permeated quantities across the whole of the experimental time $[0, t_n]$ was calculated. Afterwards, respective AUC_0^t values were calculated by means of a trapezoidal rule for the whole time values interval.

Continuous permeation profiles were obtained with two alternative methods as follows:

Numerical interpolation. A cubic spline method, implemented in the Inplot program v.4.03 (Graphpad Software Inc., 1992), was used for this purpose. This method is able to define sufficient number of points for the nonmodelistic description of each tabulated series of cumulative permeated amounts vs. time (Q vs. t.).

Fitting of a sigmoidal mathematical equation. A sigmoidal equation (Eq. 8) was fitted to the experimental results:

$$Q = \frac{Q_{max} \cdot t^{\gamma}}{t^{\gamma} + t_{Q50}^{\gamma}} \quad (8)$$

and corresponding parameters were calculated:

- Asymptotic value of the function (Q_{max}).
- Time required to reach the diffusion of half of Q_{max} (t_{Q50}).
- Sigmoidicity (γ). Factor related to the shape of the curve.

Parameters were calculated by means of a non-lineal regression program (Winnonlin v. 1.1, Scientific Consulting Inc.) in order to achieve the best possible solution.

This function was used merely in an attempt to obtain an accurate mathematical description of the experimental curves and for its selection, three possible equations were previously tested: Hyperbolic function, Sigmoidal function and Sigmoidal with a lag-time. The sigmoidal resulted the best descriptive of the experimental points. This equation is frequently used in pharmacokinetic studies and is useful for describing different biologic processes.

It is known that calculation of Q vs. t profiles with splines is clearly influenced by experimental error, resulting sometimes in spurious oscillations in the cubic spline calculation. In fact, the numerical derivative of Q vs. t generated a graphic profile with local maximums and minimums that limited severely the descriptive value of the results. Those problems were obviated with the sigmoidal fitting. Nevertheless, for both options, calculations were performed in order to obtain the corresponding AUC_0^t values.

- Numerical derivative of the relation Q vs. t taken as a measurement of the permeation rate of drug across the skin (dQ/dt). For each spline numerical interpolation or sigmoidal equation result, the numerical derivative was calculated with the aid of a calculation sheet. Thus, for each permeation profile a series of specific values representative of the instantaneous drug permeation rate were obtained. For each series of points, two centralization measures were taken: the mean (Mean dQ/dt) and the median (Mdn dQ/dt).

Goodness of Calculations

For each calculation approach, agreement between experimental results (Q_{24}obs) and predicted values (Q_{24}cal) was assessed by means of:

- Determination coefficient (R^2) of regression between experimental and predicted points on each permeation experiment.
- Error estimation of prediction at t = 24 h (%Q_{24}error). It was calculated as described in Eq. 9:

$$\%\ Q_{24}\ \text{error} = \frac{Q_{24}\ \text{cal} - Q_{24}\ \text{obs}}{Q_{24}\ \text{obs}} \qquad (9)$$

Estimation of Terpene Efficacy

In order to quantify the increase in permeation achieved, and given the high inter-individual variability of these studies (12), the calculated values of each parameter were standardized by dividing them by the reference value obtained under identical conditions but in the absence of an enhancer (control), as commonly used.

For evaluation of statistical differences among the five terpene formulations, a non-parametric multiple comparison test (Scheffé) was run for the series of ratios using the SPSS®program v.11.0.1 (Spss Inc.).

RESULTS

Analytical Method

Accuracy, expressed as relative error percentage, gave a maximum value of 7%. Precision, as variation coefficient percentage of interpolated concentrations of reference solutions, ranged between 2.9% and 10.7%. Chromatographic suitability at 0.03 μg/ml proved satisfactory. Within the concentration interval, the analytical method was found to be satisfactorily lineal.

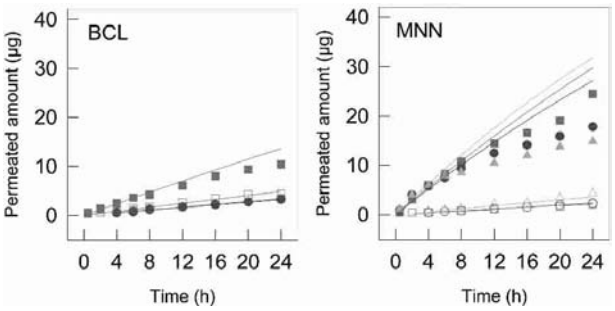

Fig. 1. Individual permeation profiles of alprazolam for BCL and MNN experiments. Symbols indicate the experimental values, and respective lines describe the permeation profile predicted by the finite-dose equation. Respective controls are included (filled symbols: enhancer curves; open symbols: control curves).

Alprazolam Solubility

Given the lack of published data on the solubility of alprazolam under the working conditions used in the current study, we proceeded to determine its saturation concentration at 32°C in pH 7.4 buffer. The mean value resulting from these measurements was 86.64 ± 7.43 μg/ml (n = 12).

Permeation Experiments

Figure 1 shows the alprazolam permeation profiles obtained for BCL and MNN formulations and its respective control. Experimental permeated amounts of drug are represented with respective fitted curves of the finite dose model.

Figure 2 presents the same experimental results with corresponding sigmoidal fitting.

Median values of P_1, P_2, Kp, J_{max} and Q_{24}cal of alprazolam obtained in control conditions for each tested formulation are shown in Table II. This table includes also the mean values of the group.

Tables III and IV present the values of the permeation parameters of alprazolam for each enhancer formulation obtained respectively for the diffusional method and for the

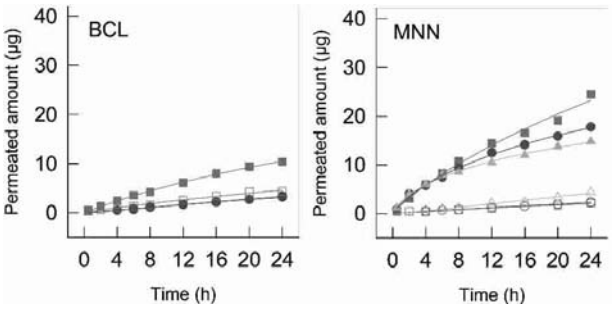

Fig. 2. Individual permeation profiles of alprazolam for BCL and MNN experiments. Symbols indicate the experimental values, and respective lines describe the permeation profile predicted by the sigmoidal equation. Respective controls are included (filled symbols: enhancer curves; open symbols: control curves).

Table II. Median Values of Intrinsic Permeation Parameters of Alprazolam Through Human Skin for Each Terpene Pretreatment Control (Control Cells)

Parameter	CIN	MNT & LIM	MNN	BCL	Mean	SD
$P_1 * 10^6$ (cm h^{-1})	795.00	5103.00	249.00	575.50	2365.10	2506.89
P_2 (h^{-1})	0.98	0.14	3.52	1.90	1.34	1.42
$K_p * 10^6$ (cm h^{-1})	781	554	572	892	671	157
J_{max} ($\mu g\ h^{-1}$)	0.10	0.09	0.10	0.15	0.11	0.03
Q_{24} ($\mu g\ h^{-1}$)	2.0	2.0	2.4	3.4	2.4	0.6

Mean values and standard deviations of the group are also indicated; n = 3.

non-diffusional method. Relating finite-dose fitting, convergence was achieved in all cases. Given the short number of replicates, median values instead of mean values were calculated for summarizing the results.

Concerning the permeation rate estimations, Fig. 3 shows an example for one concrete experiment that compares the theoretical evolution of transdermal flux obtained with the modelistic approach (Eq. 6) and the rate estimation from the sigmoidal fitting that is independent of the diffusional equation.

Table V summarizes the results of calculations obtained for assessing the goodness of calculations, whereas Fig. 4 displays the whole results of $\%Q_{24}$error as a function of Q_{24} value.

The respective ratios of enhanced efficacy for each permeation parameter are summarized on Table VI.

Variability of ratios as a function of parameters of formulations are represented on Figs. 5 and 6.

Based on the results of the multiple comparison test (Scheffé test), the formulations of enhancers can be classified in terms of efficacy over alprazolam transdermal permeation, in three homogeneous subgroups that are statistically different between them ($\alpha = 0.05$): (LIM, MNN-MNT, and BCL-CIN) formulation of limonene, formulations of methone and menthol, and formulation of oxabicycloheptane and cineol.

DISCUSSION

Experimental Conditions of the Permeation Study

The first experimental work was the estimation of the aqueous saturation concentration of alprazolam under our defined conditions (32°C, pH 7.4). It resulted to be 86.64 μg/ml, confirming the low solubility of the drug. This result is consistent with the findings of other authors. For example, Yalkowsky *et al.* (13) reported 114 μg/ml at 25°C in water. This value is actually very similar, and the difference can be attributed to differences in methodology and experimental conditions.

Permeation experiments were then designed to ensure sink conditions in the receptor compartment . The initial donor concentration was always 70 μg/ml, representing about 80% of the saturation concentration. Additionally, it is a concentration high enough to supply a gradient of drug in solution through the skin membrane.

Permeation Data Treatment

Mathematical treatment of experimental data aimed to obtain representative permeation parameters of the drug to quantify either the rate or the extent of permeation.

Two strategies were proposed in order to obtain information about the permeation rate:

- Transdermal flux was first calculated applying Equations 5 and 6. The finite dose model is not a stationary model and a linear relationship between permeated amounts and time is never reached. For this reason, the maximum value of flux was considered as a definite value. This parameter has been considered α by Anissimov *et al.* (14) as a clearer estimator of transdermal properties than Kp.

Table III. Finite-Dose Model Permeation Parameters of Alprazolam Through Pretreated Skin

Enhancer formulation	CIN (n = 3)	MNT (n = 3)	LIM (n = 3)	MNN (n = 3)	BCL (n = 2)
$P_1 * 10^6$ (cm)	781.11	531.41	1981.10	450.56	1222.14
	2051.62, 54.12	1458.20, 467.72	2172.07, 1020.99	520.17, 354.10	1240.76, 1203.51
P_2 (h^{-1})	5.80	14.10	13.72	23.37	1.50
	53.41, 3.43	15.14, 8.75	14,89, 4.89	35.37, 18.75	2.36, 0.63
$K_p * 10^6$ (cm h^{-1})	4530	8044	14004	10527	1845
	7036, 2890	12763, 6595	32345, 9690	12523, 9754	2933, 758
J_{max} ($\mu g\ h^{-1}$)	0.83	1.31	2.23	1.69	0.37
	1.43, 0.53	2.05, 1.05	5.18, 1.55	2.01, 1.57	0.60, 0.14
tJ_{max} (h)	0.24	0.12	0.12	0.12	0.90
	1.20, 0.12	0.12, 0.12	1.20, 0.12	0.12, 0.12	1.44, 0.36
MTT (h)	130.4	73.5	42.2	56.1	490.8
	204.3 84.1	89.6, 46.3	61.0, 18.3	60.6, 47.2	780.1, 201.6
Q_{24}cal (μg)	18.1	26.9	40.9	33.1	8.4
	30.2, 11.9	38.5, 22.2	69.6, 31.3	38.0, 31.1	13.6, 3.2

Respective median, maximum, and minimum values are reported for each enhancer formulation.

Table IV. Non-modelistic Permeation Parameters of Alprazolam Through Pretreated Skin

Enhancer formulation	CIN (n = 3)	MNT (n = 3)	LIM (n = 3)	MNN (n = 3)	BCL (n = 2)
Q_{24}obs (μg)	13.2	18.0	29.5	17.8	6.8
	23.3, 7.1	37.1, 12.8	56.2, 27.2	24.5, 17.8	10.4, 3.2
Splines approach					
AUC_0^{24} (μg h)	179	242	403	266	90
	316, 104	494, 208	844, 390	312, 235	141, 39
Mdn dQ/dt (μg h^{-1})	0.54	0.71	1.36	0.55	0.29
	0.97, 0.22	1.48, 0.50	1.91, 1.03	1.14, 0.44	0.44, 0.13
Mean dQ/dt (μg h^{-1})	0.54	0.73	1.25	0.73	0.28
	0.91, 0.27	1.56, 0.53	2.24, 1.07	1.01, 0.60	0.43, 0.13
Sigmoidal approach					
AUC_0^{24} (μg h)	182	246	407	269	91
	317, 105	501, 208	854, 393	320, 238	142, 39
Mdn dQ/dt (μg h^{-1})	0.94	1.33	2.48	1.20	0.55
	1.76, 0.47	3.05, 0.96	3.84, 1.90	1.78, 0.88	0.84, 0.27
Mean dQ/dt (μg h^{-1})	1.07	1.47	2.49	1.47	0.57
	1.90, 0.58	3.07, 1.10	4.69, 2.13	1.94, 1.22	0.88, 0.27
Q_{24}cal (μg)	12.8	17.7	29.9	17.6	6.9
	22.8, 7.0	36.9, 13.2	56.3, 27.4	23.2, 14.7	10.5, 3.2
Q_{max} (μg)	54.4	62.8	117.1	64.9	23.7
	73.2, 29.4	86.6, 19.8	146.2, 97.3	116.4, 34.7	37.2, 10.2
t_{Q50} (h)	99.5	53.8	64.9	64.9	54.1
	122.4, 103.9	120.0, 12.6	100.3, 42.7	1192., 37.0	62.8, 45.4
γ (μg h^{-1})	0.82	1.02	0.88	0.80	1.10
	0.93, 0.72	1.13, 0.85	1.06, 0.76	0.87, 0.71	1.24, 0.97

Respective median, maximum, and minimum values are reported for each enhancer formulation.

Median values of maximal intrinsic flux ranged between 0.09 and 0.15 μg/h (equivalent to 0.04–0.06 μg cm^{-2} h^{-1}). These values are two to three times lower than those reported by Carelli *et al.* (15) using an aqueous saturated gel of alprazolam through human skin (0.12 μg cm^{-2} h^{-1}). The difference does not seem to be relevant given that they used a saturated donor formulation vs. our finite dose experiment, and also given the high variability usually found in this type of studies. Based on these results, we assume that the other finite dose permeation parameters (e.g., K_p and MTT), are affected by the same error, due to the relationship between flux and permeability coefficient.

Relating MTT, it proved not to be amodelistic but dependent on the diffusion model. Thus, this parameter is related to the value of K_p and inversely proportional to it.

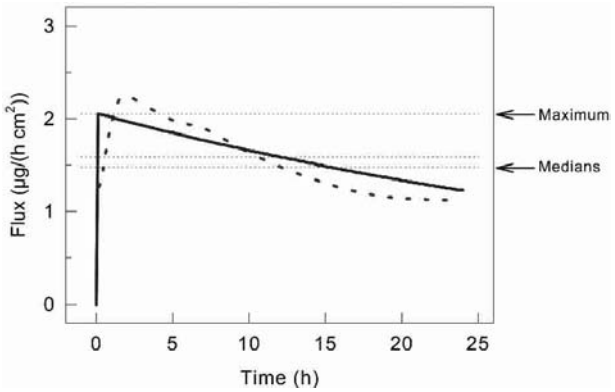

Fig. 3. Example of the evolution of the transdermal flux calculated from the finite-dose model (solid line) or from numerical derivative dQ/dt (dotted line). Maximum predicted flux (J_{max}) is indicated and also the median values of J and dQ/dt.

- An alternative method for estimating the permeation flux involved the use of the series of instantaneous permeation rates. For estimating the overall permeation rate, the median value of instantaneous rates of drug permeation (Mdn dQ/dt) was considered the best estimator of a central measure of each set of values instead of the maximum value.There is no reason to assume a normal distribution of values (Mean dQ/dt), and when respectives mean and median of dQ/dt are compared, slight differences between them are observed. Obviously, differences appear because dQ/dt variations are not linear and a marked decrease is observed at initial times, just after pretreatment, as it can be observed in Fig. 3.

Median values of flux after skin pretreatment ranged between 0.37 and 2.23 μg/h using the finite dose model approach and between 0.55 and 2.48 μg/h by means of the sigmoidal approach. Although the range of values is quite similar, given the lack of predictivity of the finite-dose diffusion model of the experimental values at a given point in time, and having also observed erratic oscillations in the numerical interpolation using the spline method, the best method of estimation of permeation rate resulted to be the non-modelistic calculation using the fitting of a sigmoid function. This option constitutes a useful calculation method which is not restricted by the conditions of the finite-dose model.

A measure of the extent of permeation was made according to the value of the area under the curve of the drug permeation profile (AUC_0^t). Although our review of the literature suggests that this parameter does not apply to transdermal permeation studies, we believe that it provides useful information about the amount of drug which permeated the

Table V. Summary of Measurements of Goodness Estimations for the Diffusional Treatment and the Sigmoidal Approach

Parameter	CIN (n = 3)	MNT (n = 3)	LIM (n = 3)	MNN (n = 3)	BCL (n = 2)	Control
R^2 (diffusional)	0.9851	0.9798	0.9948	0.9687	0.9932	0.9748
Q_{24} error (diffusional)	46.5	42.2	26.9	62.9	16.0	1.3
R^2 (sigmoidal)	0.9957	0.9977	0.9979	0.9946	0.9969	0.9744
Q_{24}error (sigmoidal)	–0.9	0.3	0.9	–2.3	0.4	1.7

Mean values of R^2 and percentages of Q_{24}error are shown for each group of experiments.

skin. As non-modelistic, its value is obviously robust. Furthermore, this parameter has resulted proportional to the experimental permeated amounts at 24 h (Q_{24}obs) and also to the amodelistic Q_{24}cal based on the sigmoidal fitting.

Concerning the sigmoidal approach, the time to reach half the asymptotic value (t_{Q50}) has limited usefulness, and sigmoidicity values cannot be assimilated to any diffusional phenomenon.

When estimators of goodness of fitting are considered, it can be clearly seen that, in absence of terpenes, the diffusional model fits adequately to the experimental data. Kp and Jmax are in a good agreement with the other approaches. Considering the results on Table IV, the determination coefficient (R^2) between experimental and calculated permeated amounts for both estimation approaches was similar (around 0.974). Also, the percentages of error in the estimation of the permeated amounts at 24 h resulted acceptable (lower than 2%). These assessments pointed to a good description of the experimental results by both methods.

Nevertheless, diffusional treatment of terpene-pretreatment experiments clearly resulted less descriptive than corresponding sigmoidal ones. The finite dose model showed lower accuracy than the sigmoidal approach: Values of R^2 ranged between 0.9687 and 0.9948 for the diffusional model while all were higher than 0.9945 for the sigmoidal approach. The same tendency was also observed in the percentage of error in Q_{24} estimation. Values were lower than 2.3% (sigmoidal) or between 16.0 and 62.9% (diffusional). Moreover, Fig. 4 illustrates that at low Q_{24} values, both approaches exhibited the same accuracy, whereas at higher Q_{24} values, the percentages of error increased considerably for the diffusional approach, confirming the overestimation.

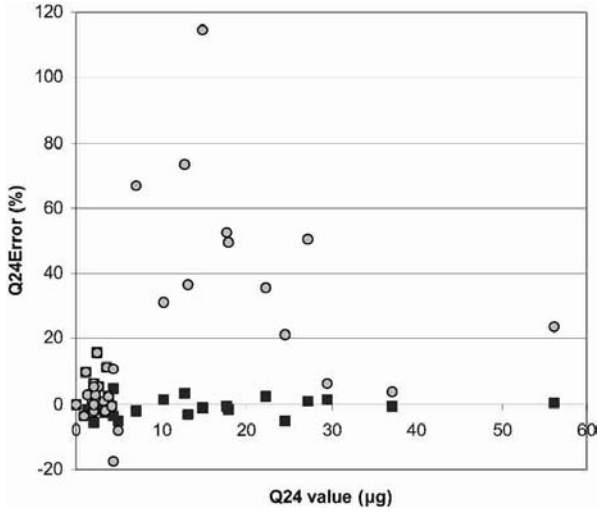

Fig. 4. Percentages of error of Q_{24} estimation (Q_{24}error) for the diffusional fitting (empty circles) and the sigmoidal approach (filled squares) independently of the pretreatment applied.

In addition, as can be seen in Figs. 1 and 2, diffusional fitting present an over-estimation mainly at the latest experimental points. This suggest a threat to the validity of the finite-dose diffusion model in this case where skin is pretreated with terpenes.

The Enhancing Effect of Terpenes on Alprazolam Permeation

Another aim of the current study was to evaluate the enhancing effect of terpenes on alprazolam skin permeation. Other authors (15) have yet reported that the intrinsic transdermal flux of this drug across human skin does not enable to predict therapeutic plasma concentrations at stationary equilibrium. Consequently, it is necessary to use permeation enhancers in order to increase this permeation level.

Terpenes, as a group of enhancers, have been considered given the reversibility of their effect on the stratum corneum. In fact, some authors (2) have reported that in pretreatment experiments with terpenes there is a continuous decline in drug flux over time, without this being attributable solely to a drastic decrease in drug concentration in the donor compartment. Thus, their disappearance from skin could explain a progressive decline in the initially improved drug's transdermal permeation.

For this group of enhancers, the fit of the finite-dose diffusion equation gave notably higher values of P_2 related with basal values, while P_1 resulted practically unaffected. Taking into account that P_2 is directly proportional to the value of the diffusion coefficient (Eq. 3), our results are consistent with those of other authors (3) and suggest that the

Table VI. Enhancement of Alprazolam Permeation After Pretreatment of Human Skin with Terpenes (Median Values of Enhancement Ratios)

Enhancer formulation	CIN	MNT	LIM	MNN	BCL
R $K_p * 10^6$	7.0	11.9	25.3	21.8	1.8
R J_{max}	6.9	12.0	25.3	17.4	1.9
R Q_{24}obs	5.1	7.6	13.7	7.7	2.0
R Mdn dQ/dt (sigmoidal)	3.4	7.3	13.6	6.3	1.7
R AUC (sigmoidal)	4.6	11.7	19.3	9.3	1.8
R Q_{max}	2.4	7.1	18.8	6.7	1.0
R t_{Q50}	0.8	0.7	2.3	0.8	0.5
R γ	0.8	0.7	0.7	0.8	1.1

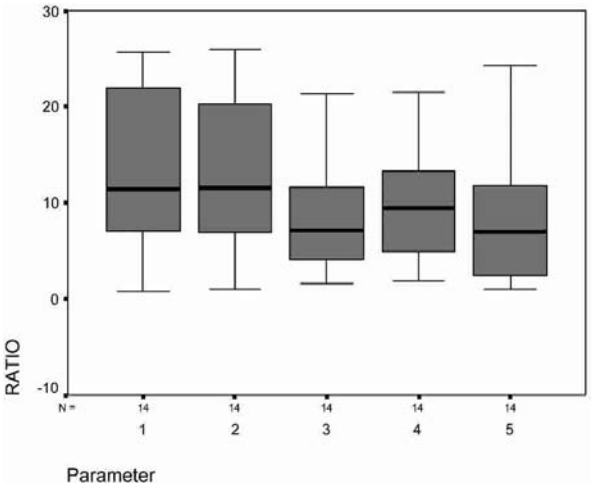

Fig. 5. Variability of the enhancement ratio values grouped by parameters (1, R K_p; 2, R RJ_{max}; 3, R Q_{24obs}; 4, R AUC; 5, R Mdn dQ/dt).

enhancing effect of terpenes isdue to an increase in the drug's diffusivity in the stratum corneum.

Some authors have described the permeation through rodent skin of d-limonene (16) or l-menthol (17) in hydroalcoholic solutions, reaching values of permeated terpene of about 2 mg/cm^2 at 8 h. In our laboratory, we have also observed the permeation of d-limonene toward the receptor compartment even during the short pretreatment period. This was demonstrated by quantifying the d-limonene in the receptor phase during the pretreatment stage. At the end of this stage (6 h), around 50 μg of d-limonene/cm^2 had diffused. Moreover, the presence of ethanol in the formulation of d-limonene facilitates nonspecifically the incorporation of terpene into skin, thus increasing the efficacy of its enhancing effect (18).

In the case of terpenes, it can be assumed that their rapid transit across skin produces a brief enhancing effect, and that the stratum corneum progressively recovers its original properties. This would explain why the description of the experimental points by a finite-dose diffusion model is sometimes unsatisfactory, as the skin permeability does not remain constant throughout the experiment.

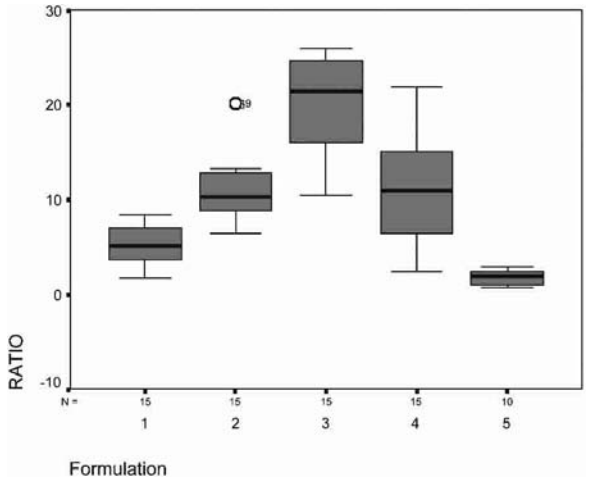

Fig. 6. Variability of the enhancement ratio values grouped by formulations (1, CIN; 2, MNT; 3, LIM; 4, MNN; 5, BCL).

Obviously, modelistic calculation assumes that the values of the partition and diffusion coefficients are truly constant throughout the permeation, able for calculation of a true permeability coefficient. If the finite dose model is valid, the MTT of the drug is the best parameter to illustrate this effect. But in our case, only a rank order is useful for evaluation, resulting that the lower value of MTT corresponds to higher values of permeated amounts at 24 h.

So then, we believe that the non-modelistic parameters obtained from a sigmoidal curve fitting are a valid approach for quantifying the extent and rate of drug permeation, and even the enhancing effect of substances, such as terpenes, that are rapidly cleared from skin.

Anissimov and Roberts (19) have recently described a diffusion model for percutaneous penetration to study the effects of either a variable diffusion coefficient or a variable partition coefficient in the stratum corneum over the diffusion path length. They have demonstrated that penetration experiments data for an heterogeneous membrane can be perfectly described also by modeling assuming an homogeneous membrane. What is reported in our case is a modification on coefficient values over the experimental time assuming that penetration enhancers substances are cleared from the different layers of the skin. A mathematical description has still not been reported, and surely the explanations of those authors can be helpful for this purpose.

With respect to the enhancement ratios, the values derived from t_{Q50} and sigmoidicity do not prove useful, as they are highly dependent on the shape of the permeation curve. Ratios derived from K_p, J_{max}, Mdn dQ/dt, AUC, Q_{24}obs and Q_{max}, they are all equally suitable for classify the rank order of efficacy of those terpene formulations.

Considering the statistical analysis, although normality of data couldn't be discarded (Shapiro-Wilk's test), given the low number of replicates for each experiment (only triplicates), and that data variability depends on the parameter considered (see Figs. 5 and 6), it was decided to apply a more conservative non-parametric test instead of an ANOVA. Differences with basal values were not studied because the variable of study was the ratio of enhancement. The results of the multiple comparison test classify the formulations in three homogeneous subgroups that are: LIM, MNN-MNT, and BCL-CIN. Those results allow to stablish a rank order of efficacy of the formulations, from d-limonene/etanol (the highest) to bicycloheptane or cineol (the lowest), with menthol and menthone the intermediate. The formulation of d-limonene contained very high amounts of ethanol enabling a drug permeation rate over 15-fold higher than that obtained under basal conditions. Similarly, it presented a short time required to reach maximum flux, while the drug remained in the skin for a comparatively shorter time. However, the values of K_p and J_{max} gave a notably higher value than expected, attributable to the limitations of the finite-dose model in this particular case. In fact, ethanol was added to the formulation for its known solubilizing effect and –what is more important- because it also diffuses rapidly through the skin (16,20), and it could show a synergistic effect with terpenes.

Although the efficacy of terpene formulations has been demonstrated, this methodology is not applyable in real-life situations. The pretreatment constitutes an *in vitro* experimental procedure for evaluation of enhancers and measuring an effect to be modulated afterwards in formulation studies.

In summary, the results obtained in this study illustrate the limitations of applying the generally useful finite-dose diffusion model to experiences of pretreatment of skin with substances presenting a rapid transdermal diffusion. Permeation parameters independent of the diffusion model may be a suitable approach as they don't depend on the mechanistic constraints of the finite-dose model.

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