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Research paper

Collaborative permeation of drug and excipients in transdermal formulations. *In vitro* scrutiny for ethanol:limonene combinations

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ABSTRACT

Enhancement of skin permeation of drugs is affected by the simultaneous co-permeation of excipients that hinder the predictivity of *in vitro* tests. The collaborative effects of two permeation enhancers (ethanol and d-limonene) of a lipophilic drug (alprazolam) have been simultaneously assessed in human skin under different *in vitro* conditions: integrated setups of diffusion cell experiments with selective concentration gradients of permeants (asymmetric) or without (symmetric) have been combined with coadministration dosages (*all-in-one*) at different concentrations or short-time skin pretreatment to scrutiny this mutual performance.

Findings: Drug permeation is increased under moderated supersaturation but reaches a stationary level above 33 % of its solubility. Ethanol in absence of a concentration gradient increases *ca.*5 times basal drug permeation. Limonene until 20 % permeates human skin proportionally to its donor concentration but its effect does not depend on ethanol in symmetric conditions and is based on skin imbibition rather than on a carry-on effect. Simultaneous permeation of ethanol and limonene reaches a stationary state after 1.5 h, enough time to achieve maximal enhancement of alprazolam permeation. Additive enhancement is based on ethanol solubilisation maximized by skin saturation of terpene. Complementary analyses of skin disruption published in the literature are in line with these assessments and consolidate them.

1. Introduction

Skin permeation of drugs for transdermal administration usually requires strategies of chemical enhancement and/or appropriate formulation techniques to reduce the epidermic barrier-function with an acceptable reversibility and skin tolerance [1,2] to achieve systemic therapeutic efficacy [3].

When a transdermal formulation is applied on skin, all of its components can be released and even absorbed, resulting in mutual interactions among permeants that are not easily reproducible with single *in vitro* experiments but with a dermatopharmacokinetic approach [4]. It is required to know the specific permeation profile of each substance to assess its collaborative performance in the whole formulation.

It is commonly postulated that terpenic compounds profusely facilitate skin permeation due to its structural similarity with native lipids of *stratum corneum* [5,6]. As a consequence, terpenes would reduce the lag time of drug permeation by an increase in drug diffusivity through the epidermis [7]. Other studies demonstrate [8] the synergistic effect of terpenes with short chain alcohols (e.g. ethanol, glycols) over the permeation of certain insoluble drugs based on *in vitro* experiments investigating the mutually-influenced flux values of drug and these other key-components of the formulation [9,10].

There are evidences of rodent-skin permeation of d-limonene or l-menthol in hydro alcoholic solutions [8,11]. Additionally, the release and human skin permeation of d-limonene from TTS matrix prototypes has been also inferred [7]. Under a mechanistic and toxicological point of view, other authors [6] have indirectly assumed the progressive penetration of cyclic terpenes in the viable epidermis/dermis in few hours and its fast clearance from stratum corneum. However, comprehensive direct measurements of permeated levels of limonene:ethanol through human skin are not available in the literature.

These facts also address to an important safety issue. Monoterpenic enhancers are present in numerous cosmetic products and have been used traditionally to facilitate the administration of active substances through skin. Some authors conceive them as non-irritant enhancers due to its natural origin, its ubiquity in fragrances and traditional uses [12,13]. Otherwise, those substances have been quoted as potential contact-allergens [14,15] and other authors evidence their probable

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overabsorption if applied to immature skin [16] or alert to their toxicity if administered on certain mucosae [17]. Furthermore, they have an unpredictable toxicokinetic, being incompletely and slowly eliminated in urine or lungs [18]. As a result of this controversy, European legislation [19] demands its declaration in the composition of cosmetic products whenever they are present. Furthermore, they are rarely included in the manufacture of transdermals, even though they are indeed active as transdermal permeation enhancers [20].

Mutual influences between co-permeants can be investigated running complementary *in vitro* setups with saturated vs solubilized formulations. In addition, bearing in mind that the passive diffusion of a substance at equal concentrations at both sides of the skin (symmetric disposition) is negligible, transmembrane concentration differences generate a concentration gradient of each asymmetrically-set substance [21]. Thus, permeation fluxes of permeants following Fick's first law of diffusion can be selectively generated *in vitro* by means of specific asymmetric setups.

Considering, additionally, the mode of application of the formulation components, two additional options of *in vitro* setup are suitable: whether simultaneous coadministration of drug and enhancers on the skin (imitating the common application of a transdermal or locally-acting product) other the sequential application of an enhancer formulation during a preliminary time previous to drug administration. This two-step approach is suitable to compare the effects of high concentrations of enhancers and/or the effect of skin-saturation with enhancer series.

This investigation seeks to provide practical and usable information, based on usual *in vitro* diffusion tests, about the collaborative influence of ethanol and a lipophilic terpene (d-limonene) over the rate and extent [22] of *in vitro* human skin permeation of a moderately lipophilic drug (alprazolam) which is known to be markedly enhanced by them.

2. Material and methods

Three series of human-skin permeation tests have been designed to discriminate the mechanistic roles of different permeants. The experimental conditions are summarized in Table 1. The denominations of symmetric – asymmetric setups has been taken from Kurihara_Bergstrom et al. 1991 [21] when they remark that the passive diffusion of a substance at equal concentrations at both sides of the skin (symmetric disposition) is negligible. In our case, we have assumed that all transmembrane concentration differences generate a concentration gradient of the respective asymmetrically-set substances. Based on this idea, we have intended to study the mechanism of ethanol dissociating the contribution of its permeation through skin (asymmetric setup, Series III) and the effects of skin imbibition (symmetric setup, Series II) taking the Series I as a drug permeation baseline (absence of ethanol).

2.1. Materials

d-limonene and methyl caprylate were purchased to Sigma chemical (Steinheim, Germany). Ethanol, methanol, propylene glycol (PG) and sodium and potassium phosphates were purchased to Merck (Barcelona, España). Alprazolam was gently donated by former Upjohn Farmoquímica (Madrid, España) and diazepam was purchased to Acofarma (Barcelona, España). Demineralized water (conductance $\leq$ 70μS/cm) was obtained on-site for all the experiments.

2.2. Methods

2.2.1. Permeation experiments

Vertical diffusion cells [23] with a *ca.*13 mL or 10 mL receptor volume and a 2.54 cm^2 permeation area have been used to set up coadministration and skin pretreatment experiments respectively. Abdominal female human skin (26 to 58 years old) was obtained *ex vivo* from leftovers of abdominal plastic surgery. The protocol of skin residues donation was approved by the Bioethics Committee of the "Hospital de Barcelona" (Hospital de Barcelona, SCIAS, Barcelona, Spain). After chirurgical excision, skin remains were frozen at −20 °C and skin sections (400 μm thickness) were obtained using an electric Dermatome (Aesculap GA 630, Tuttlingen, Germany). The day before, skin specimens were passively defrosted overnight at 4 °C and tempered at 32 °C. Except for the experiments with ethanol 30° (series II) a six-cells automated sampler apparatus (Microette, Hanson Research, USA) has been used to obtain 0.7 mL samples (CV < 0.5 %). Volume replacement was done in all cases.

All laboratory samples were cautiously preserved to avoid evaporation losses of excipients.

2.2.1.1. Coadministration of drug and enhancers. Two series of symmetric experiments have been run using, as donor and receptor solvent, whether 1/15 M Sorensen pH 7.4 buffer phosphates [24]or ethanol 30° aqueous solution (see Table 1). Donor formulations have consisted of different dispersions of d-limonene and drug at saturation levels according to an infinite dose diffusion model. Final concentration of alprazolam was in all cases equal to 5 mg/mL. A blank of d-limonene and three concentration levels in buffer and also in ethanol (above its theoretical solubility in each vehicle) have been tested. Experiments lasted for 30 h and the contents of donor phase were replaced [25] every-eight hours with a fresh volume of each continuously-stirred respective dispersion.

(1) Coadministration in pH 7.4 buffer phosphates. Dispersions of alprazolam and d-limonene donor solutions were prepared 24 h before the experiment and maintained in continuous stirring overnight until the initiation of the experiment: Alprazolam powder was dispersed in 35 mL buffer phosphates at 32 °C (175 mg/35 mL) adding, on each case, the required amounts of d-limonene to achieve concentration levels of 0.0, 0.25, 1.0 or 2.5 %. One mL of each respective saturated dispersion was used as donor phase. Randomized skin samples of six different donors

Table 1
Setups of permeation experiments (series I to III). Composition of donor and receptor phases and permeants that have been analized in each study.

Series	Application of drug and enhancers	Drug dose	Donor phase (dose)	Receptor solvent	Solvent symmetry	Analyzed permeants
I	Coadministration	infinite	Alprazolam 5 mg Limonene 0,0.25,1.0,2.5 % Buffer pH 7.4	Buffer pH 7.4	Symmetric	drug, terpene
II	Coadministration	infinite	Alprazolam 5 mg Limonene 0,0.5,5.0,10 % Ethanol:Water 30:70	Ethanol: Water 30:70	Symmetric	drug, terpene
III	Pretreatment	finite	*1st step:* Limonene 20 % Ethanol:PG 80:20 *2nd step:* Alprazolam 105 μg Buffer pH 7.4	Buffer pH 7.4	Asymmetric with respect to ethanol	drug, terpene, ethanol

were used in each experiment. Baseline results were obtained in an additional six-cells experiment using a 5 mg/mL saturated solution of alprazolam without d-limonene.

(2) Coadministration in ethanol 30° (v/v). Permeation of drug and terpene at the levels of d-limonene: 0, 0.5, 5.0, 10.0 % were assessed. Donor phases were prepared in the same way as the former coadministration study by overnight dispersing Alprazolam in ethanol 30° at 32 °C (175 mg / 35 mL) and adding d-limonene up to the indicated proportions.

On each experiment, skin was used from an unique individual. Three cells contained the formulation under evaluation and the other three cells were used as a basal reference without limonene.

2.2.1.2. Skin pretreatment experiments. The epidermal sides of skin specimens set in vertical diffusion cells [23] were pretreated by application of 200 μL dissolution of d-limonene in ethanol/ PG (20:64:16 w:w:w) during 6 h [26]. Receptor solution was pH 7.4 buffer phosphates [27]. Afterwards, the limonene solution was rid off and skin surface was rinsed with increasing dilutions of PG: buffer pH 7.4. Baseline levels were obtained with additional skin specimens of the same origin but placing buffer phospates in the donor compartment during 6 h instead of the limonene pretreatment solution. Thereafter, 1.5 mL alprazolam solution (70 μg/mL in pH 7.4 buffer phosphate) was placed in the donor compartment to evaluate drug permeation through pretreated skin.

Skin samples proceeded from three different individuals. Along the 6 h pretreatment, samples of receptor compartment were taken every hour and further 0.5 and 2.0 h after pretreatment finalization, yet in the drug permeation phase. In all those samples, ethanol and limonene concentrations have been quantitated to estimate their respective permeation profiles.

After skin pretreatment, drug permeation was monitored by 700 μL sampling of receptor solution at 0.5, 2, 4, 6, 8 h, and at fixed 4 h intervals until the final time of 24 h.

2.2.2. Assay of permeants

Only the substances asymmetrically distributed were analysed (see Table 1). Thus, d-limonene and alprazolam were investigated in all three studies whereas ethanol was only analysed in the pretreatment experiments (series III).

From the whole laboratory sample (700 μL), aliquots of 200 μL were generated for each different analysis. Sample vials for d-limonene or ethanol assay were immediately sealed and cooled after sampling. Cross-contamination of samples was monitored [28] by the goodness of blanks along the analytical series, and also by the accuracy of the quality controls.

2.2.2.1. Analysis of d-Limonene. Terpene concentrations in receptor samples have been analyzed with gas chromatography and mass spectrometry with electronic impact ionization (GC/MS-EI).

Analytical samples from each experiment (aqueous or hydro-alcoholic) were prepared by extraction with *n*-hexane. For this purpose, 150 μL laboratory sample was combined with 300 μL internal standard solution in *n*-hexane. Extraction was done with agitation (30 min) and centrifugation (4000 rpm/10 min/ 5 °C). From upper (organic) phase, 50 μL was taken for the assay.

Analyses were run with an HP 5890 Gas chromatograph coupled with an HP 5971A Mass selective detector (Hewlett-Packard, Geneva, Switzerland). Stationary phase was a Methylsilicone with 5 % Phenylmethylsilicone column (Agilent HP-5®). Sample volume was splitted till 2 μL. Temperature was raised from 50 °C (13.5 min) to 300 °C at 40 °C/min and stabilized during 2 min. Methyl caprylate was used as internal standard at 800 ng/mL. Spectrometric ionization was performed at 70 eV and detection was done at 93 *m/z* (d-limonene) or 87 *m/z* (internal standard).

Calibration was based on the mean values of four intra-assay curves. Seven concentrations of d-limonene in *n*-hexane at 5 °C were prepared ranging from 50 to 2500 ng d-limonene/mL. Dilutions were extracted combining 300 μL of each standard dilution with 150 μL of either pH 7.4 buffer or 30° ethanol. Additionally, control samples using an intermediate concentration of d-limonene were run every fifteen test samples. On each experiment, precission and accuracy was evaluated and considered satisfactory if lower than 10 %.

2.2.2.2. Analysis of ethanol. Ethanol quantitation in the pretreatment-phase samples of series III was also performed with GC/MS-EI. 150 μL samples were combined with 300 μL *n*-Hexane, long-time agitated and centrifuged (4000 rpm/10 min/5°C). 50 μL supernatant was taken for the assay. Gas chromatography was developed through a Methylsilicone with 5 % Phenylmethylsilicone column (Agilent HP-5®). Sample volume was splitted till 2 μL. Temperature was raised from 40 °C (4 min) to 85 °C at 40 °C/min and stabilized during 4.5 min. Finally, temperature raised till 200 °C at 40 °C/min. Spectrometric ionization was performed at 70 eV and detection was done at 31 *m/z* for ethanol.

Calibration was performed with an external standard of ethanol using the mean values of four intra-assay curves of ethanol treated in identical conditions, between 500 and 10000 μg/mL. Inter-assay variability was not calculated.

2.2.2.3. Analysis of alprazolam. Alprazolam was quantitated by means of a validated HPLC/UV method (222 nm). Samples were eluted over a C18-reversed phase column (NovaPak C18 5mmx10mm, 4 μm, Waters®) with Methanol:Water (60:40) at 2 mL/min [27]. Calibration was done with seven standard levels between 0.03 and 10 μg/mL. An internal standard (diazepam) was used for eventual sample reanalyses.

Standards and real permeation samples were prepared combining 200 μL standard solution, or a 200 μL aliquot of the laboratory sample, with 50 μL 1.6 μg/mL diazepam solution. Weighted linear regression was applied given that the distance between the maximum and minimum value was larger than two orders of magnitude [29].

Accuracy was expressed as the relative error at each concentration. Precision was expressed as the RSD of different replicates [28] Maximum mean error was + 6.6 % (at low concentrations) and maximum RSD resulted to be 2.3 % and considered acceptable. Concentrations from areas below 0.03 μg/mL were considered as non-quantifiable.

2.2.3. Calculation of continuous permeation profiles

The cumulative permeated amounts (Q) vs time (t) profiles were calculated based on respective cell volumes and sample volume (700 μL) corrections.

Attempting to describe univocally all the experimental curves of all the groups of experiments, a sigmoidal fitting (Eq. (1)) was used to plot a continuous numerical description [22] during the whole experimental time [0, t_n]:

$$Q_t = \frac{(Q_{max})\cdot t^{\gamma}}{t^{\gamma} + t_{Q50}^{\gamma}} \quad (1)$$

where Q_{max} is the likely asymptotic value, t_{Q50} is the time required to reach the diffusion of the half value of Q_{max} and the exponent γ was the sigmoidicity, a shape-related coefficient. Best parameter values were obtained by means of non-linear regression using Nelder-Mead minimizations algorithm (WinNonlin v 3.4, Pharsight software).

Resultant individual curves were used to to obtain an accurate mathematical description of the experimental curves. Afterwards, respective $AUC_0{}^t$ values were calculated by means of a trapezoidal rule. Mean values of these curves of permeated alprazolam, limonene or ethanol were plotted grouped by each replicates level.

2.2.4. Calculation of permeation parameters

2.2.4.1. Extent and rate of permeation:.

- Permeated amounts at the end time (Q_t): Q_{24} for coadministration experiments, Q_6 for pretreatment phase and Q_{24} for drug permeation after pretreatment. In all cases, calculated from each sigmoidal fitting.

- Area under the permeated amounts/time (Q vs t) curves (AUC_0^t). It was calculated by means of a trapezoidal rule using the whole duration divided in 25 equidistant points. Areas were truncated at 24 h for coadministration and after-pretreatment phase and at 6 h for the pretreatment phase.

- Skin permeation rate (dQ/dt) estimated as the numerical derivative of each calculated sigmoidal curve using the respective 25 calculated points of the trapezoidal calculation [22] with a conventional calculation worksheet. Then, its respective median was taken as a measurement of centralization (Mdn dQ/dt) because a normal distribution of values cannot be assumed. Thus, for each replicate of permeation profile, an estimation of drug permeation rate was obtained regardless the shape of the curve.

2.2.4.2. Terpene efficacy. Comparison of results of each set of replicates was standardized to evaluate limonene concentration effects and also to reduce the inter-individual variability [30]. The enhancement of drug permeation in each series was expressed with the enhancement ratio ("ER") dividing each parameter by its corresponding value obtained under identical conditions but without enhancer (mean control).

2.2.4.3. Collaborative permeation of enhancers. The extent of simultaneous permeation of limonene (L) and ethanol (E) during pretreatment was evaluated calculating the individual ratios (μg/g) of permeated amounts at each time ("Ratio L/E") as in Eq. (2)

$$Ratio\ L/E = \left(\frac{Q_{permeated\ LIM} * 1000}{Q_{permeated\ EtOH}}\right) \quad (2)$$

All ratios were plotted as a whole and grouped by each level of replicates. In this case, graphical description was achieved fitting to the ratios the asymptotic curve described in Eq. (3):

$$Q_t = Q_0 + (Q_{max} - Q_0)*(\frac{t}{t + t_{Q50}}) \quad (3)$$

Statistical differences (alpha = 0.05) between the permeation parameters of each set of replicates were evaluated with a non-parametric multiple comparison test (Scheffé) using the SPSS_program v.11.0.1 (Spss Inc.).

3. Results

Figs. 1 to 5 depict the mean permeation profiles of each permeant in each group of experiments. Symbols describe the corresponding mean values and the respective lines are the sigmoidal curves from Eq. (1) fitted to each series of symbols for a better graphical description. Variabilities are reported in Tables 2 to 4 and further considered in statistical analysis. The highest variability was observed in the pretreatment experiments (Series III).

All profiles were convex except for alprazolam with buffer (concave). Lag time could only be observed for this case. Concerning the heterogeneity of the resultant curves due to mutual influences between the permeation enhancers, the morphology of permeation profiles reveals the non-existence of a proper lag time. This value only exists properly in the experiments with buffer and without enhancers. For this reason, calculation of this parameter has not been considered as achievable.

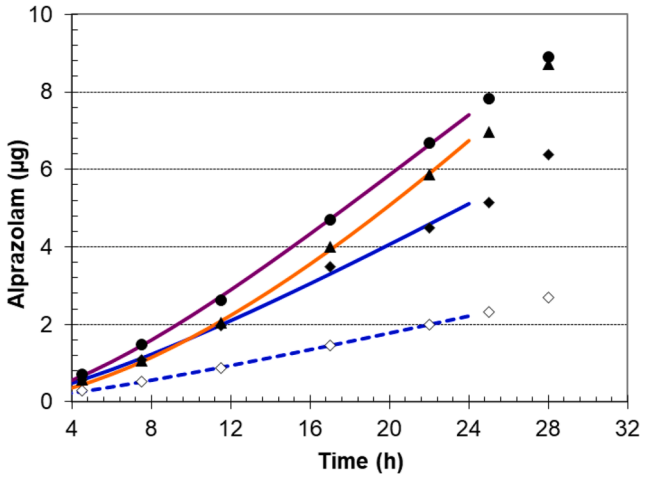

Fig. 1. Mean permeation of alprazolam during coadministration study in pH 7,4 buffer (Series I). Diamonds (0.25%), triangles (1.0%), circles (2,5%). Basal profile is represented with the dotted line and voided diamonds.

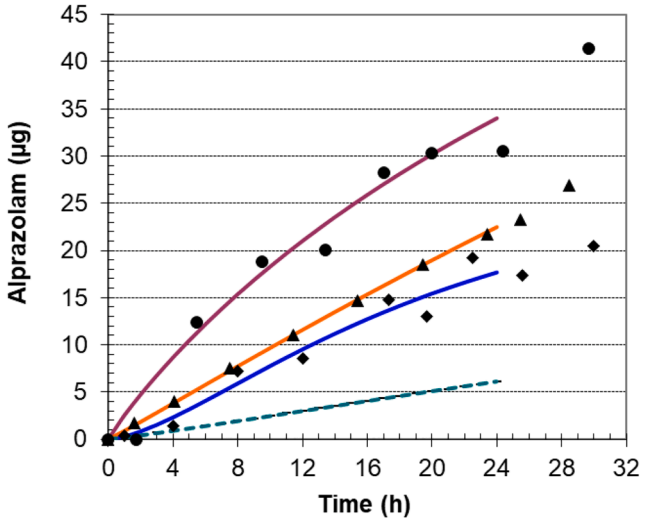

Fig. 2. Mean permeated amounts of alprazolam in coadministration with ethanol 30° (Series II). Diamonds (0.5%), triangles (5.0%), circles (10.0%). Mean basal curve of the whole replicates is represented with the dotted line.

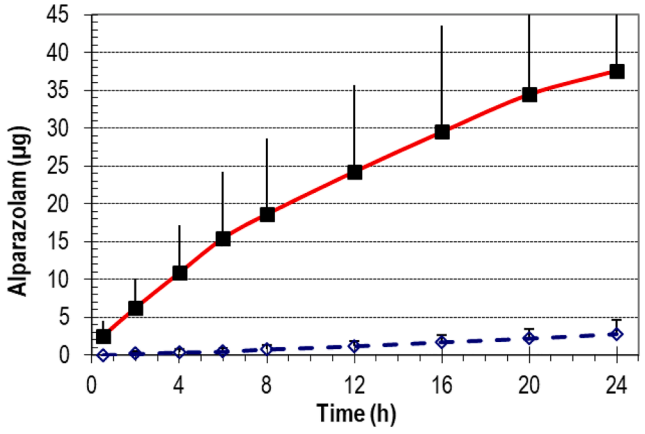

Fig. 3. Mean alprazolam permeation profiles (with SD) after a 6 h pretreatment with a d-limonene/ ethanol solution (Series III). Squares). Voided diamonds indicate the corresponding drug permeation across an only buffer-pretreated skin.

Table 2
Permeation parameters of Alprazolam in the symmetric Buffer pH 7.4 experiments (Series I): Permeated amount at 24 h (Q24), Area under the permeation curve truncated at 24 h (AUC024) and Median of the instantaneous slopes (Median dQ/dt). Mean, standard deviation in parenthesis at the first line. ER in italics for each case in the second line.

Experiment	Q 24 (μg)	AUC_0^{24} (μg.h)	Median dQ/dt (μg/h)
0.25 % limonene	3.84 (2.20)*	39.86 (23.31)*	0.18 (0.10)*
	1.7	*1.7*	*1.8*
1.0 % limonene	7.45 (3.17)	75.16 (35.11)	0.36 (0.15)
	3.4	*3.2*	*3.6*
2.5 % limonene	6.76 (2.82)	62.52 (30.41)	0.33 (0.14)
	3.0	*2.6*	*3.3*

*Statistical differences with the other two groups.

Table 3
Permeation parameters of Alprazolam in the symmetric Ethanol 30° experiments (Series II): Permeated amount at 24 h (Q24), Area under the permeation curve truncated at 24 h (AUC024) and Median of the instantaneous slopes (Median dQ/dt). Mean, standard deviation in parenthesis in the first line. ER in italics for each case.

Experiment	Q 24 (μg)	AUC_0^{24} (μg.h)	Median dQ/dt (μg/h)
0.5 % limonene	18.82 (2.49)	229.89 (48.82)	0.85 (0.13)
	3.1	*3.2*	*3.2*
5.0 % limonene	22.47 (6.18)	274.79 (77.58)	0.94 (0.25)
	3.7	*3.9*	*3.5*
10.0 % limonene	33.78 (1.03)*	486.51 (41.39)*	1.14 (0.17)*
	5.5	*6.8*	*4.2*

*Statistical differences with the other two groups.

Table 4
Permeation parameters of Alprazolam in the pretreatment experiment (Series III): Permeated amount at 24 h (Q24), Area under the permeation curve truncated at 24 h (AUC024) and Median of the instantaneous slopes (Median dQ/dt). Mean, standard deviation in parenthesis. Mean ER for each parameter (in italics).

Experiment	Q 24 (μg)	AUC_0^{24} (μg.h)	Median dQ/dt (μg/h)
Buffer (no enhancer)	2.66 (1.73)	29.74 (18.2)	0.12 (0.08)
20 % limonene / EtOH /PGL	37.53 (16.24)	555.53 (258.8)	1.86 (1.8)*
ER	*15.7*	*19.8*	*12.0*

*Statistical differences with the group of 10% in coadministration (Series II).

3.1. Alprazolam permeation

Mean permeation profiles of alprazolam in coadministration studies with buffer phosphates and ethanol 30° are plotted in Figs. 1 and 2 grouped by d-limonene levels and including the respective basal permeation (no terpene).

Fig. 3 depicts the alprazolam permeation profiles through pretreated skin with the hydroalcoholic d-limonene solution and also through non-pretreated skin (no terpene).

Respective permeation parameters of alprazolam are summarized on Tables 2 to 4. Basal permeations of Series I and III (pH 7.4 buffer) are not statistically different between them but statistically lower than results of Series II (ethanol 30°) in absolute terms.

Statistical differences between permeation extent parameters (Q24 and AUC_0^{24}) of alprazolam with buffer in coadministration (Series I) have been observed between the lower concentration level of d-limonene (0.25 %) and the middle and upper levels (similar). In the experiments using ethanol 30° (serie II), statistical differences of Q24, AUC_0^{24} and dQ/dt have been observed between the lower and middle concentration levels and the upper concentration level (10 %), which is greater.

Statistical differences of ER with buffer phosphates are, similarly, observed between the 0.25 % d-limonene level and the 1.0 % and 2.5 % levels. With ethanol 30°, statistical differences are observed between the 10 % level (the highest) and the lower ones 0.5 and 5.0 % (similar).

3.2. Limonene and ethanol permeation

Mean permeation profiles of d-limonene in each coadministration series are plotted in Figs. 4 and 5.

Permeation levels of d-limonene and ethanol in the pretreatment phase are plotted in Fig. 6.

In this case, individual values are plotted for an accurate explanation of its variability. The corresponding individual ratios of limonene / ethanol permeation levels are plotted in Fig. 7.

Permeation parameters of limonene in coadministration experiments are summarized on Tables 5 and 6.

Permeation parameters of limonene and ethanol during pretreatment are summarized on Table 7.

4. Discussion

Alprazolam is a psychotropic drug indicated for the treatment of moderate or severe anxiety states and anxiety associated with depression. It should not be used to treat mild anxiety or tension associated with the stress of everyday life. It is only indicated when the disorder is severe, disabling or subjecting the individual to extreme distress. [63]

Model-permeants of this set of experiments have been selected as follows: Alprazolam is a suitable transdermal candidate [31]; it is commonly administered by oral route, with an absolute bioavailability around 80 %, mainly due to hepatic first-pass metabolism. Daily oral doses range from 1 to 4 mg/day. Given its short pharmacokinetic half-life (12.5 h), [64] immediate release tablets must be administered each 8 to 12 h. As a result, oral modified release products, instead of immediate release, are majoritarily used in chronic treatments. All these biopharmaceutical properties, joined with a favourable partition coefficient (logP = 2.50), [65][66],[67] point this drug as a good candidate for transdermal administration [31] which requires the use of penetration enhancers to achieve therapeutic drug levels [27]. Sesquiterpenes, e.g.: d-limonene, are effective enhancers of alprazolam [22] and other moderately hydrophobic permeants, [32,33] and ethanol is a solvent-type enhancer commonly combined with terpenes to achieve additive effects [34]. It is commonly assumed that terpenes act disrupting intercellular lipids and/or keratin domains and tight junctions and, thus, facilitating drug solubility in skin lipids.[60] Wang et al.[68] demonstrate that polar terpenes act interfering with hydrophilicity by destroying the tight network of ceramide, and loosening lipophilic areas and keratin of SC. In this work, their simultaneous human skin

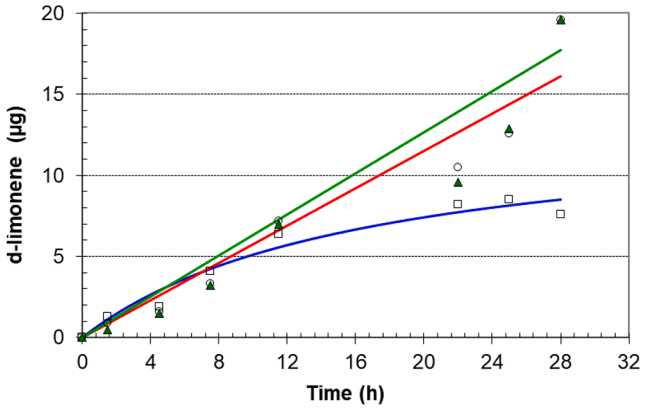

Fig. 4. Mean d-limonene permeation levels in the coadministration experiment with pH 7.4 buffer. Voided squares: 0.25%, filled triangles: 1.0%, voided circles: 2.5%.

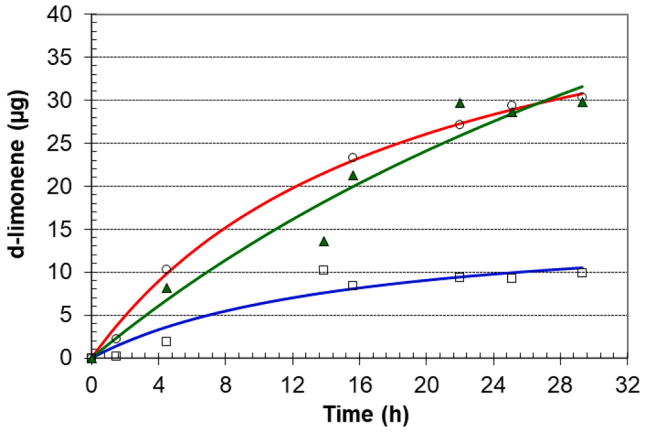

Fig. 5. Mean d-limonene permeation in the experiment of coadministration with ethanol 30°. Voided squares: 0.5%, voided rounds: 5.0%, filled triangles: 10.0%.

permeation has been scrutinized integrating a set of discriminative *in vitro* results.

4.1. Alprazolam permeation

Its basal flux (0.12 µg/h) is similar to results reported by other authors [31]. Mean Q24 levels are about the 14 % of drug solubility in pH 7.4 buffer phosphates and probably express a saturation of gradient diffusion towards aqueous receptor compartment (near *sink* level). There are significant differences between this basal value and counterpart results with ethanol 30° (Series II) due to the contribution of ethanol inside the skin over the permeation of alprazolam.

Lag time could be calculated only for the buffer coadministration curves, that followed a proper fickian diffusion, but not for ethanol and pretreatment curves that showed a convex shape attributable to a severe modification of skin properties by the presence of ethanol as permeable substance and the rapid skin clearance of terpene (see Fig. 1 vs Fig. 2).

Standardised enhancement ratios (ER) of drug permeation are low and similar in both series of coadministration (see Tables 2 and 3). Their similarity informs that the effect of the concentration of d-limonene does not depend on the presence of ethanol in skin. However, in absolute terms, and as observed also with basal permeations, alprazolam has permeated much more in presence of ethanol than when using only an aqueous buffer solution. In fact, parameters from ethanol coadministration (Series II) are significantly higher than those with buffer coadministration (Series I) in a ratio of *ca.* 4:1 (4.3 for Q24, 5.7 for AUC and 3.3 for dQ/dt). Similarly, Morimoto et al. (1993) found marked differences between water and Ethanol 40 % in the permeation of Morphine ClH (log P octanol: water 1.42 at physiological pH) [35]. It can be confirmed that the presence of ethanol increases proportionally the absolute permeation of the drug although the percentage contribution of d-limonene seems to be independent of the presence of ethanol (no synergistic).

Other authors [11,23] have postulated that ethanol facilitates the unspecific incorporation of terpene into skin. In our case, both coadministration series demonstrate the same relative effect (ER) of limonene towards alprazolam permeation, but the stronger effect of ethanol series is attributable to a mechanism of skin accumulation and modification of skin properties because the experiment has been set up under symmetric conditions for this substance. It is noted that the concentration of ethanol is unable to completely dissolve the amounts of d-limonene and vehiculize it.

The maximum flux of alprazolam (1.86 µg/h = 0.73 µg/cm^2/h) has been achieved in the series III experiments. Enhancement ratios are the highest, and this formulation and mode of administration (pretreatment with d-limonene/ethanol/PG at 20:64:16 w:w:w) is the most effective.

Table 5
Permeation parameters of d-Limonene in the symmetric Buffer pH 7.4 experiments (Mean and standard deviation): Permeated amount at 24 h (Q24, µg), Area under the permeation curve truncated at 24 h (AUC024, µg.h) and Median of the instantaneous slopes (Median dQ/dt, µg/h).

Experiment	Q 24 (µg)	AUC_0^{24} (µg.h)	Median dQ/dt (µg/h)
0.25 % limonene	8.08 (1.80)	127.59 (31.20)	0.21 (0.11)
1.0 % limonene	12.82 (3.81)	129.12 (40.99)	0.45 (0.24)
2.5 % limonene	11.61 (4.76)	147.10 (69.12)	0.50 (0.23)

The extent parameters (Q24 and AUC_0^{24} values) of Series II at 10 % and Series III at 20 %, are not statistically different between them, but flux (dQ/dt) is statistically higher (see Table 4).

Although this *in vitro* set up is not suitable in clinical practice, it allows to demonstrate the contribution of the skin imbibition with enhancers over the permeation enhancement.

4.2. d-limonene permeation

Although some author's [36] have not inferred its *in vitro* transdermal passage, current results with human skin have directly evidenced the rate and extent of d-limonene permeation in all the experimental conditions, even during the short skin pre-treatment.

Donor concentrations of d-limonene in coadministration experiments (series I and II), are as usual as in TTS reservoir-type prototypes [37]. They fall above its aqueous solubility and, thus, all formulations are oversaturated. In this sense, Moser et al. (2001) [38] observed a proportionality between permeation rates of a lipophilic molecule and its degree of supersaturation (for 1 to 4 times higher than its solubility). In our case, ethanol 30° experiments (series II), have shown differences between the 0.5 % level (33-times its water solubility) and both 5.0 and 10.0 % levels (333 and 666 times), which are extremely higher than solubility and, in practice, similar between them.

The concordance between alprazolam and d-limonene permeations in both coadministration studies is not proportional to the nominal concentrations, as shown in Figs. 4 and 5. A practical estimation of the implications of limonene permeation can be done indirectly in terms of its effects over alprazolam permeation. Thus, in Series I (pH 7.4 buffer), alprazolam permeation extent (Q24) and rate (dQ/dt) at the lower d-limonene level (0.25 %) are not statistically different than the corresponding basal results. It suggests that limonene permeation and/or penetration at this low.

concentration (see Table 5) is not effective over alprazolam permeation. Otherwise results are statistically different from the respective middle and upper concentration levels (1.0 % and 2.5 %).

Similarly, in Series II (ethanol 30°) permeation rate and extent of middle and upper levels (5.0 % and 10.0 %) were similar between them but different from the lowest level (0.5 %).

Concerning pretreatment (Series III), an infinite dose of d-limonene has assured a constant thermodynamic activity of permeants in solution. Limonene permeated at 6 h (shown in Fig. 6) represent only the 0.35 % of the total donor amount, satisfying *sink* conditions.

Summarizing, limonene permeation at 6 h is directly proportional to donor concentration, being 4.72 µg/cm^2 if dispersed at 10 % in ethanol 30° and 56.13 µg/cm^2 if dissolved at 20 % with ethanol:PG 64:16 (see Fig. 5 and Table 7). Considering the solubility of d-limonene in ethanol 30°, Q24 values at 5 % and 10 % levels are quite coincident representing a 1.46 and a 1.48 % of the solubility and less than a 0.06 % of the donor amounts (worst case) supporting the same oversaturation effect. Results are lower than values reported by others. With human epidermis, Cal & Janicky [7] found about 1500 µg/cm^2 d- limonene at 8 h after application of a matrix type formulation and Morimoto et al. [39]obtained higher *in vitro* permeation levels through rodent skin (e.g. menthol in hydroalcoholic solutions): about 2000 µg /cm^2 at 8 h.

Skin uptake of terpenes has been related sometimes with its

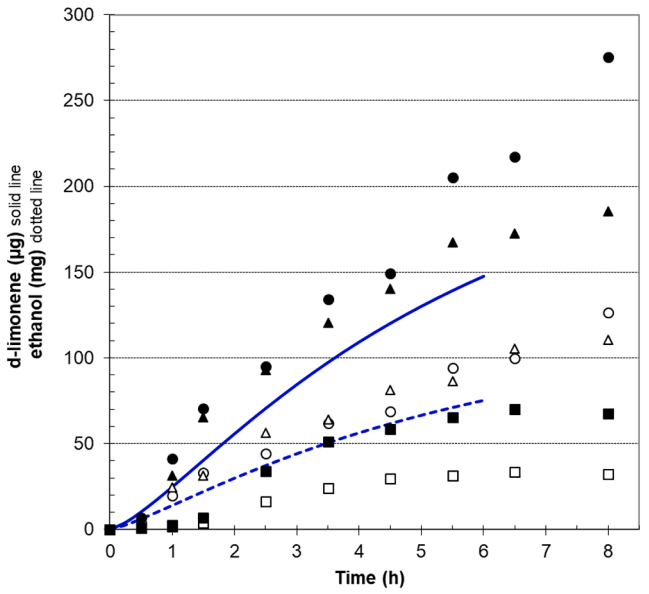

Fig. 6. Individual permeation profiles of d-limonene (µg, filled symbols, solid line) and ethanol (mg, voided symbols, dotted line) in the pretreatment study (Series III). Each experiment is depicted with a different symbol.

Table 6
Permeation parameters of d-Limonene in the symmetric Ethanol 30° experiments (Mean and standard deviation): Permeated amount at 24 h (Q24, µg), Area under the permeation curve truncated at 24 h (AUC024, µg.h) and Median of the instantaneous slopes (Median dQ/dt, µg/h).

Experiment	Q 24 (µg)	AUC_0^{24} (µg.h)	Median dQ/dt (µg/h)
0.5 % limonene	9.90 (4.42)	158.97 (66.75)	0.26 (0.12)
5.0 % limonene	28.51 (3.48)	420.73 (82.15)	0.95 (0.02)
10.0 % limonene	28.93 (7.79)	442.63 (128.53)	0.90 (0.24)

Table 7
Permeation parameters of d-limonene and ethanol during the pretreatment experiments. (Mean and standard deviation): Permeated amount at 6 h (Q6), Area under the permeation curve truncated at 6 h (AUC_0^6) and Median of the instantaneous slopes (Median dQ/dt).

Permeant	Q 6	AUC $_0^6$	Median dQ/dt
d-limonene	142.58 (70.0) µg	479.08 (242.7) µg.h	23.40 (10.1) µg/h
ethanol	72.99 (35.6) mg	249.03 (124.6) mg.h	11.46 (5.1) mg/h

corresponding enhancement effect [40,41]. Given its high logP 4.579 ± 0.243 [42,43], a high dermal accumulation of d-limonene [36] and a low degree of permeation are expectable. Then, if the permeation of d-limonene is limited by its lipophilic skin retention it can be infered that, at both highly supersaturated donor dispersions (5 % and 10 %), skin capacity has reached a limit value and, thus, similar permeation values at the medium and high concentrations are obtained although donor concentrations are initially different.

Additionally, after the pretreatment and ridding off the limonene solution (6 h), its transdermal flux remained unaffected two hours later, suggesting that a remarkable degree of skin saturation was achieved. In fact, Cornwell et al. (1994) [43] estimated a SCR uptake of *ca. 90 mg/g* (dry weight), a massive amount of d-limonene able to be distributed in the intercellular lipid domain and also in the protein domains.

Concerning convex kinetics of permeation, no lag-time can be estimated as the skin properties do not remain constant throughout the experiment [22]. Reversibility of the terpenes effect is related with this disappearance from skin, most probably by an absorption into deeper skin layers. In fact, reversibility has been explained by means of a competitive bonding to native lipids in conjunction with a slow skin clearance [43,44]. In this sense, bioavailability of essential oils assess the degree and how fast are absorbed after their topical administration and their elimination routes and kinetics [45] as other chemical families of enhancers are rapidly and mostly metabolized and/or excreted [46] too.

After dermal application, limonene and other monoterpenoids seem to be readily absorbed with a short absorption half time of 0.05 h and maximum plasma levels reached 6.3 min after application [47]. Similarly, Obata et al. (1993) [40] obtained the maximum enhancing effect of limonene over the diclofenac rat skin permeation yet after only 1 h of pretreatment with 1 % limonene dispersed in ethanol 40 %.

4.3. Ethanol effect over drug permeation

The hydroalcoholic formulations of terpenes are commonly used in experimental TTS formulations, [40,48,49]such as ethanol 66 % with terpenes 5 %, whether for water soluble [50] or lipophilic drugs [51,52]. In fact, d-limonene is practically insoluble in water but its solubility in ethanol 30° reaches near 150 µg /mL [40] without significantly altering the drug saturation solubility.

In some cases, a direct relationship between ethanol and drug concentrations in rat skin has been observed [53] and, thereafter, between drug in skin and drug in plasma. It suggests that permeation levels of ethanol should be proportional to drug skin permeation, as has been shown with some NSAIDs [40,54].

The effect of ethanol over the permeation of d-limonene or alprazolam has been investigated firstly under symmetric conditions with respect to pH 7.4 buffer or ethanol 30° (see Table 1). Based on these results, it can be assumed that the enhanced permeation of alprazolam and limonene, previously discussed, are not due to the neat skin permeation of ethanol but to its own skin accumulation.

Under asymmetric conditions, ethanol flux from the pretreatment solution has ranged around 4.51 mg/cm^2.h (11.46 mg/h) attaining a maximum permeated amount at 6 h of 28.74 mg/cm^2 and demonstrating also its massive permeation through human skin (*ca.*75 mg during 6 h). In similar conditions, but with hairless mouse skin, Shirakura et al. [55] achieved permeation levels proportionally to its donor concentration. After 6 h, they achieved cumulative levels of 50 mg/cm^2 from a 25 % ethanol solution and 110 mg/cm^2 from a 50 % solution, attaining the maximum permeation with limonene dispersed in 65 % ethanol. Similarly, Okabe et al.(1992) [54] obtained same order rat skin permeation values from a solid 60 % ethanol acrylic gel patch, ranging around 20 to 45 mg/cm^2 at 8 h.

Concerning the use of PG in pretreatment experiments (series III), glycols show additive effects when added to terpenoids too [3,56,57]. PG does not significantly increase terpene delivery to the *stratum corneum* above that provided by application of neat terpenes [43] and probably disrupts also the orientation within the lipid bilayer [51]. Conversely, transcutol does not appear to modify the stratum corneum structure, but acts on the concentration of drug in the corneum aqueous zones maximizing its thermodynamic activity [58,59].

4.4. Collaborative mechanisms of permeation

Many hypothesis concerning the mechanism of action of limonene: ethanol combinations appear in the literature, most of them limited by the particular reach of the experimental investigation. Some of them priorize the effect of *drug-pull* across the skin and others reinforce the effect based on skin accumulation and the SCR disorganization [60].

Permeation profiles of both enhancers in asymmetric conditions during pretreatment (n = 3, plotted in Fig. 6) are certainly variable but the corresponding ratios limonene / ethanol (Fig. 7) clearly show a homogenous tendency that reach a constant asymptotic value after *ca.* 1.5 h, gently before 6 h. When drug has been thereafter applied, transdermal flux of enhancers is yet stationary (Ratio L/E about 2 µg/g) and

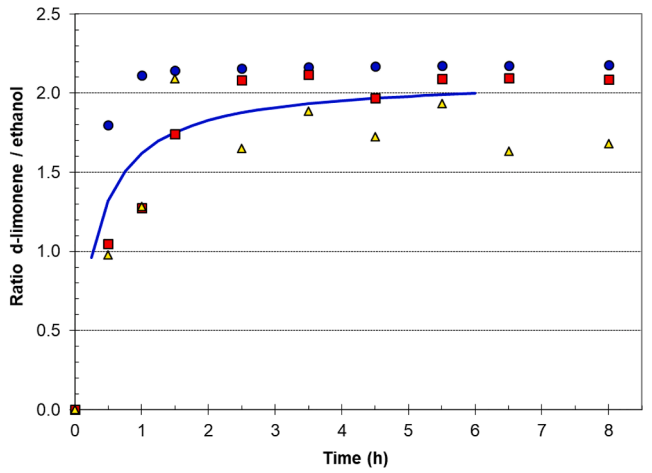

Fig. 7. Individual ratios (d-limonene / ethanol) permeated amounts grouped by experiment as in Fig. 6 (circles, squares, triangles). Asymptotic curve (pooled data) is predicted and drawn with the solid line.

enhancement at maximum achievable levels. Analogously, this short-time saturation period is reported in the literature by other authors, [6](i.e.: menthone, 4 h, human skin). Anyway, it reveals a faster permeation in rate and extent of ethanol *versus* limonene that can be explained by its higher diffusivity and that could facilitate the subsequent limonene permeation by increasing its diffusion coefficient [21].

As a possible enhancement mechanism of the combination of d-limonene with ethanol, some authors postulated that d-limonene penetrates into the skin under coexistence with ethanol and may change the barrier structure of the stratum corneum. The transfer of ethanol to the skin is thereby enhanced under the coexistence of d-limonene in the skin. Thus, drug permeation can be promoted due to its affinity with ethanol resulting in a close association between the amount of ethanol in the skin and the percutaneous absorption of a lipohilic drug (log P = 4.3). [69]

Conversely, our results suggest the opposite, i.e. ethanol permeation is more rapid and extensive than d-limonene (see Fig. 7). This fact, justifies the wide enhancing success that this solvent provides when combined with a broad list of other types of chemical enhancers.

Shirakura et al. (1995) [55] demonstrated, also with an asymmetric setup, a direct proportionality between d-limonene mouse permeation and the ethanol donor concentration: after 6 h, they reported d-limonene levels of 1400 μg/cm^2 from a 25 % ethanol solution and about 2700 μg/cm^2 from a 50 % solution. Simultaneous permeation of both enhancers at different concentrations returns also quite regular ratios L/E: 28 and 24 μg/g for 25 % and 50 % ethanol respectively. Differences with present results with human skin suggest [30] also a possible major relative diffusion of ethanol in mouse skin.

The dramatic increase of flux values (dQ/dt) of d-limonene observed in Series III (20 % pretreatment) compared with 2.5 % limonene in Series I (e.g. 23.4 μg/h ≫>≫ 0.50 μg/h) is not proportional with the respective flux values of alprazolam (1.86 μg/h > 0.33 μg/h) reported in Tables 3 and 4. It seems reasonable to postulate that the enhancing effect of d-limonene dissolved in ethanol 64 % is not mainly due to its skin permeation rate because this over-increase is not proportional to drug permeation.

Another evidence supporting this postulate is that, although the flux of ethanol after the pretreatment tends to zero, the great enhancement of drug permeation during the 24 h later can only be attributed to the resident ethanol and, thus, to other mechanisms than its neat permeation.

Although Takayama & Nagai, 1994 [53] postulated that the cooperative effect of both enhancers could be more due to its diffusion following a concentration gradient than to its skin structure interposition, our results with ethanol at both sides (symmetric, Series II), in absence of a neat concentration gradient, demonstrate, however, that the enhancement of the combination is higher (4x) than when using limonene alone, probably because solvent-enhancers can force conformational changes of terpenes [70]indeed.

Similarly, Cornwell et al. (1994) [43] observed that the addition of ethanol to limonene does not significantly increase the human SCR uptake of d-limonene. This influence over alprazolam permeation can be inferred comparing with buffer results in Tables 2 to 4. In fact, the inclusion of terpenes inside the intercellular lipids of *stratum corneum* increases permeants diffusivity [5,43,44,61]. Only in case of highly lipophilic drugs, this rate-transport limiting-step changes from diffusion across SC to partitioning at the SC-viable epidermis interface [62]. This partition raises due to the presence of terpenes yet dissolved in ethanol rather than its direct effect themselves.[71]

After these insights in the relationship between drug permeation and enhancers permeation, additional experiment with lower concentration of enhancers or/and their direct quantitation inside the skin would be additionally useful to confirm current results. Additionally, for a transdermal prototype a more reproducible manufacturing procedure could be more tolerable and easily scalable.

Summarizing, based on current results, the most plausible mechanism is that ethanol permeates abdominal human skin rapidly and massively and its enhancing activity is due mostly to skin imbibition that facilitates the subsequent permeation of limonene and also the extent of drug permeation 3 to 5 times in comparison with its permeation in absence of ethanol with a mechanism not due to a direct pull-effect across the skin but to the modification of skin properties by enhancer imbibition.

Our results evidence that limonene truly permeates human skin with a finite flux which explains its reversible activity. Limonene permeates skin above its saturation in the vehicle either in the presence or absence of ethanol and reaching in both cases a limit value. This fact confirms that limonene applied in solutions close to saturation permeate differently from net terpenes.

This combination of enhancers over human skin reaches a stationary permeation as has also been reported in the literature with other susbtances. In this case around 1.5 to 2.0 h after its application suffices to guarantee their maximum enhancement activity.

The permeation of alprazolam, as a lipophilic model drug, is directly but not linearly proportional to the permeation of d-limonene. Its permeation is enhanced by a mechanism involving skin imbibition rather than carry-on transport.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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