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Evaluation of in vitro percutaneous absorption of lorazepam and clonazepam from hydro-alcoholic gel formulations

Carmelo Puglia [a,*], Francesco Bonina [a], Giuseppe Trapani [b], Massimo Franco [b], Maurizio Ricci [c]

[a] *Department of Pharmaceutical Sciences, School of Pharmacy, University of Catania, Viale Andrea Doria no. 6, 95125 Catania, Italy*

[b] *Department Farmacochimico, School of Pharmacy, University of Bari, Via E. Orabona no. 4, 70125 Bari, Italy*

[c] *Department of Drug's Chemistry and Technology, School of Pharmacy, University of Perugia, Via del Liceo no. 1, 06100 Perugia, Italy*

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Abstract

Clonazepam and lorazepam are two anxiolytics, antidepressant agents, having suitable features for transdermal delivery. The objectives of this study were to evaluate the in vitro percutaneous absorption of these drugs through excised human skin (stratum corneum and epidermis, SCE) and to determine their in vitro permeation behavior from a series of hydro-alcoholic gel formulations containing various enhancing agents. The best permeation profile was obtained for both drugs applying them together with Azone in combination with propylene glycol (PG): these enhancers were able to increase the clonazepam and lorazepam percutaneous fluxes at steady-state about threefold, compared to the free enhancer formulations (Control). To explain the mechanism of the used promoters, the benzodiazepine diffusion and partitioning coefficients from the gel containing the enhancers were calculated. The results indicated that the Azone in combination with PG could act by increasing the benzodiazepine diffusion coefficients, Transcutol increased only the SC/vehicle partition coefficients, limonene in combination with PG appeared to increase both partition and diffusion coefficients moderately, while PG did not increase both the parameters. Furthermore, to evaluate the potential application of tested benzodiazepine formulations containing Azone in combination with PG using the flux values from the in vitro experiments, the corresponding steady-state plasma concentrations (C_{SS}) were calculated. The obtained calculated C_{SS} values are within the lorazepam therapeutic range and suggest that transdermal delivery of this drug could be regarded as feasible. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Transdermal delivery; Lorazepam; Clonazepam; Penetration enhancers; In vitro

* Corresponding author. Tel./fax: +39-095-222258.
E-mail address: boninaf@mbox.unict.it (C. Puglia).

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1. Introduction

In recent years, the development of transdermal dosage form designed to have systemic effects has been attracting increasing attention, due to the several advantages that this administration route offers, such as a better control of blood levels, a reduced incidence of systemic toxicity, an absence of hepatic first-pass metabolism, etc.

Drug delivery via the skin is not a simple task. The outermost, and least permeable, layer of the skin, the stratum corneum (SC), is a formidable barrier both to water transport out of the body and to inward chemical permeation. In fact, the majority of drugs do not appear to penetrate the skin at a rate sufficiently high for therapeutic efficacy and only the most potent ones with appropriate physicochemical characteristics are valid candidates for transdermal delivery.

Recently, a number of benzodiazepines are being introduced for anxiolytic, hypnotic, and antiepileptic purposes. Since therapy using these drugs often involves long-term administration, the transdermal route of drug administration would have a potential merit over conventional dosage form.

To evaluate the benzodiazepines transdermal ability, different authors studied their in vitro percutaneous absorption. Touitou, for instance, studied the skin permeation profiles of midazolam maleate and diazepam through hairless mouse skin from various solvent systems (Touitou, 1986), besides Hori et al. investigated the enhancement of diazepam penetration through rat and hairless mouse skin in vitro (Hori et al., 1991) by *n*-nonane, *n*-nonanol and different monoterpenes. Carelli et al. determined the enhancement effects of oleic and linoleic acid on the skin permeation of alprazolam through hairless mouse skin (Carelli et al., 1992). Other authors studied the percutaneous absorption of clonazepam from a series of alcoholic-gel or emulsion-gel formulations containing various enhancing agents (Mura et al., 1989, 1997; Ogiso et al., 1989) using artificial membranes (Mura et al., 1989, 1993, 1996) or natural ones (rabbit ear skin) (Ogiso et al., 1989; Mura et al., 1996).

Notwithstanding this copious literature, until now, we have not found in vitro data concerning the percutaneous absorption fluxes of benzodiazepines through excised human skin. Such data could consent a better prediction of in vivo benzodiazepine transdermal administration in humans (Scott et al., 1986a) compared to other natural or synthetic membranes.

In this study, in order to evaluate the feasibility of benzodiazepine transdermal administration, we evaluated the in vitro percutaneous absorption through excised human skin of lorazepam and clonazepam from hydro-alcoholic carbomer gels. Both these drugs present a series of biological characteristics such as high first-pass metabolism, side effects, dose size and need for repetitive dosing, all of which make these drugs valid candidates for transdermal administration.

Furthermore, we assessed the ability of some penetration enhancers, such as Azone (in combination with PG), Transcutol, D-limonene (in combination with PG) and PG in increasing in vitro percutaneous absorption of these drugs.

2. Materials and methods

2.1. Materials

Clonazepam and lorazepam were supplied by Aldrich. [^{3}H]water (spec.act. 5 mCi/ml) was obtained from Amersham (UK). Carbopol 934 (Carbomer) was supplied by Biochim (Italy), Azone was a gift from Whitby Research Inc. (Richmond, VA), propylene glycol (PG) was obtained from Sigma Chemicals (St. Louis, MO), Transcutol (diethylene glycol monoethyl ether) was a gift from Gattefossè (France), D-limonene was obtained from Carlo Erba, Italy. All other materials were of analytical grade.

2.2. Preparation of hydro-alcoholic gel formulation

The composition of the benzodiazepine gels (**A**–**L**) used in this study is reported in Table 1. Hydro-alcoholic gels were prepared by dispersing 1% w/w Carbopol 934 (Carbomer) in water and

then adding an alcoholic solution in which were solubilized the benzodiazepine (1% w/w) with constant stirring. The dispersion was then neutralized and made viscous by the addition of triethanolamine. The enhancers were dissolved in ethanol or in water depending on their solubility. The gels were stored at room temperature for 24 h under air-tight conditions prior to use.

2.3. Skin membrane preparation

Samples of adult human skin (mean age 36 ± 8 years) were obtained from breast reduction operations. Subcutaneous fat was carefully trimmed and the skin was immersed in distilled water at 60 ± 1 °C for 2 min (Kligman and Christophers, 1963), after which stratum corneum and epidermis (SCE) were removed from the dermis using a dull scalpel blade. Epidermal membranes were dried in a desiccator at $\approx 25\%$ relative humidity. The dried samples were wrapped in aluminum foil and stored at 4 ± 1 °C until use. Preliminary experiments were carried out in order to asses SCE samples for barrier integrity by measuring the in vitro permeability of [^{3}H]water through the membranes using the Franz cells described below. The value of the permeability coefficient (P_m) for tritiated water was found to be $1.6 \pm 0.2 \times 10^{-3}$ cm/h which agreed well with those for tritiated water reported by others using human SCE samples (Bronaugh et al., 1986; Scott et al., 1986b).

2.4. In vitro skin permeation experiments

Samples of dried SCE were rehydrated by immersion in distilled water at room temperature for 1 h before being mounted in Franz-type diffusion cells supplied by LGA (Berkeley, CA). The exposed skin surface area was 0.75 cm^2 and the receiver compartment volume was of 4.5 ml.

The receptor compartment contained a water–ethanol solution (50:50), to allow the establishment of the 'sink condition' and to sustain permeant solubilization (Touitou and Fabin, 1988a), was stirred and thermostated at 35 ± 1 °C during all the experiments.

Approximately, 100 mg of each gel (**A**–**L**) was placed on the skin surface in the donor compartment and the latter was covered with Parafilm. Each experiment was run in duplicate for 36 h using three different donors ($n = 3$). At intervals, samples (200 μl) of receiving solution were withdrawn and replaced with fresh solution. The samples were analyzed for benzodiazepine content by high-performance liquid chromatography (HPLC) as described below. Benzodiazepine fluxes through the skin were calculated by plotting the cumulative amount of drug penetrating the skin against time and determining the slope of the linear portion of the curve and the χ-intercept values (lag time) by linear regression analysis. Drug fluxes (μg/cm^2 per h), at steady-state, were calculated by dividing the slope of the linear portion of the curve by the area of the skin surface through which diffusion took place.

Table 1
Lorazepam and clonazepam gel composition (% w/w)

Constituents	Gel code									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**L**
Lorazepam	1	1	1	1	1	–	–	–	–	–
Clonazepam	–	–	–	–	–	1	1	1	1	1
Carbomer	1	1	1	1	1	1	1	1	1	1
Ethanol	30	30	30	30	30	30	30	30	30	30
Triethanolamine	1	1	1	1	1	1	1	1	1	1
Azone	–	5	–	–	–	–	5	–	–	–
PG	–	20	–	20	20	–	20	–	20	20
Transcutol	–	–	20	–	–	–	–	20	–	–
D-Limonene	–	–	–	1	–	–	–	–	1	–
Distilled water	67	42	47	46	47	67	42	47	46	47

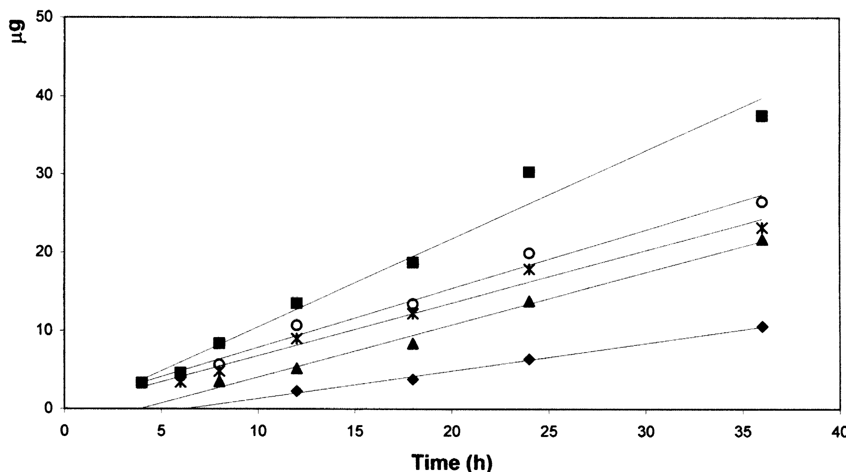

Fig. 1. Effect of different enhancers on clonazepam skin permeation from different gel formulations (F–L). (◆) Control; (■) Azone/PG; (▲) PG; (*) Transcutol; and (●) D-limonene/PG.

The effectiveness of penetration enhancers (enhancement factor, EF) was determined by comparing benzodiazepine flux in the presence and absence of enhancers:

$$\mathrm{EF} = \frac{\text{Benzodiazepine flux at steady state in the presence of enhancers}}{\text{Benzodiazepine flux at steady state in the absence of enhancers}}.$$

Statistical analysis of the data was performed using Student's t-test.

2.5. High-performance liquid chromatography

The HPLC apparatus consisted of Waters 600E system (Waters-Millipore Corporation, Milford, MA) equipped with a 20 µl loop and a Waters 486 detector. Chromatography was performed on a Lichrosphere 100 C_{18} R.P. column (particle size, 5 µm; 25 cm × 4.6 mm i.d.; E. Merck, Darmstadt, Germany). The mobile phase for lorazepam was methanol–water (60:40) and water–acetonitrile (75:25) for clonazepam. Detection was effected at 230 nm (lorazepam) or 306 nm (clonazepam). The flow rate was set at 2.5 ml/min for clonazepam and 1 ml/min for lorazepam. The retention times were of 5.66 min for clonazepam and 15.53 min for lorazepam.

3. Results and discussion

In vitro skin permeation experiments were performed using human SCE membranes instead of full-thickness skin since, as reported by others (Scheuphlein and Blank, 1973; Bronaugh and Stewart, 1984), the dermis in vitro can act as a significant artificial barrier to the absorption of lipophilic compounds.

In Figs. 1 and 2, the plots of the cumulative amounts of lorazepam and clonazepam permeated through human SCE membranes as a function of time are shown respectively.

The flux values at the steady-state of lorazepam and clonazepam from carbomer gels, calculated from the linear segments at the steady-state (Table 2), were found to be 0.48 ± 0.07 and 0.32 ± 0.06 µg/cm^2 per h, respectively.

To improve the skin permeation of examined benzodiazepines, we carried out the in vitro experiments applying these drugs together with some penetration enhancers such as limonene, Transcutol, Azone and PG extensively used to increase drug percutaneous absorption (Harrison et al., 1996; Mura et al., 2000; Almirall et al., 1996; Bonina and Montenegro, 1994a).

PG was selected on the basis of both its penetration enhancing properties and its synergic ef-

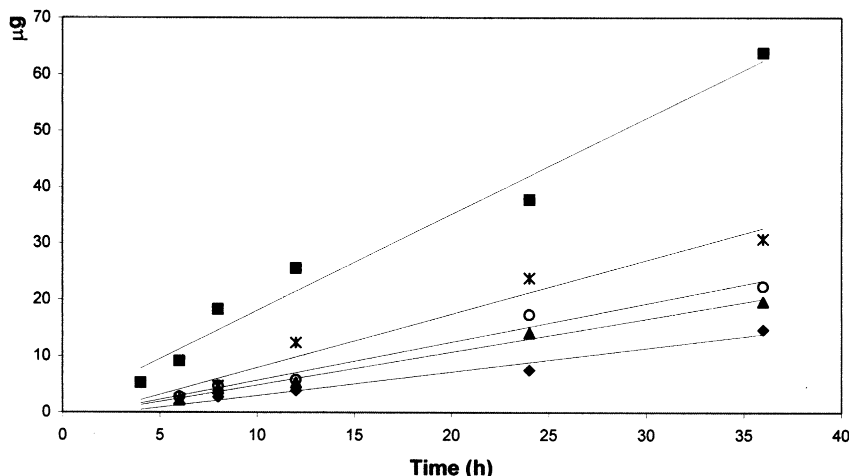

Fig. 2. Effect of different enhancers on lorazepam skin permeation from different gel formulations (A–E). (◆) Control; (■) Azone/PG; (▲) PG; (*) Transcutol; and (●) D-limonene/PG.

fect in increasing Azone and limonene enhancer activities (Zhao and Singh, 2000; Godwin and Michniak, 1999; Valenta and Wedenig, 1997; Chatterjee et al., 1997). Furthermore, in our previous papers (Bonina and Montenegro, 1992, 1994b), a PG concentration of 20% showed a higher synergic effect enhancing effectiveness of Azone.

Azone and limonene (Table 1) have been used, respectively, at 5 and 1%, since, in previous papers (Bonina and Montenegro, 1992, 1994b; Priborsky et al., 1992) these concentrations resulted suitable for an evident skin penetration ability of these substances.

In Figs. 1 and 2, the plots of the cumulative amounts of lorazepam and clonazepam permeated through human SCE membranes in presence of used penetration enhancers as a function of time are shown respectively.

From the flux values obtained (Table 2), not all the penetration enhancers tested in this study produced an increase of percutaneous fluxes of clonazepam and lorazepam compared to control formulations.

Although different works in literature (Mura et al., 1989, 1997) underlined PG ability in promoting percutaneous absorption of drugs through human skin, from the results obtained in the present study (Table 2), this enhancer appeared inefficacious when applied alone on the skin in increasing percutaneous absorption of both benzodiazepines.

Table 2
Lorazepam and clonazepam steady-state fluxes through excised human skin, lag time and EF from carbomer gels containing Azone and PG (**B** and **G**), Transcutol (**C** and **H**), D-limonene (**D** and **I**) and PG (**E** and **L**)

Gel code	Flux ± S.D.[a] (µg/cm^2 per h)	Lag time (h)	EF
A	0.48 ± 0.07	2.81 ± 0.56	–
B	1.52 ± 0.21	0.58 ± 0.11	3.16
C	0.97 ± 0.15	3.21 ± 0.64	2.02
D	0.62 ± 0.12	1.84 ± 0.36	1.29
E	0.58 ± 0.11	2.69 ± 0.48	1.22
F	0.32 ± 0.06	3.21 ± 0.64	–
G	0.96 ± 0.18	1.12 ± 0.22	2.86
H	0.55 ± 0.11	2.74 ± 0.54	1.71
I	0.68 ± 0.14	2.01 ± 0.41	2.12
L	0.48 ± 0.19	2.94 ± 0.53	1.50

[a] Each experiment was run in duplicate for 36 h using three different donors ($n = 3$). $P<0.01$: gel **B** and **C** vs gel **A**; gel **G** vs gel **F**; gel **D** and **E** vs gel **B**. $P>0.05$: gel **D** and **E** vs gel **A**; gel **I** and **L** vs gel **H**; gel **E** vs gel **D**; gel **L** vs gel **F** and **I**; gel **I** vs gel **G**. $P<0.05$: gel **B**, **D** and **E** vs gel **C**; gel **H** and **I** vs gel **F**; gel **G** vs gel **H** and **L**.

Table 3
Lorazepam and clonazepam skin permeation parameters from carbomer gels containing Azone/PG, Transcutol, D-limonene/PG and PG

Enhancer	Parameter					
	Clonazepam			Lorazepam		
	P_m ($\times 10^{-5}$; cm h^{-1})	K_{app}	D_{app} ($\times 10^{-8}$; cm^{-2} h^{-1})	P_m ($\times 10^{-5}$; cm h^{-1})	K_{app}	D_{app} ($\times 10^{-8}$; cm^{-2} h^{-1})
Control	3.2	0.36	14.7	4.8	0.24	16.8
Azone/PG	9.6	0.37	42.7	15.2	0.31	81.1
Transcutol	5.5	0.54	17.1	9.7	1.1	14.7
D-Limonene/PG	6.8	0.48	23.5	6.2	0.4	25.5
PG	4.8	0.5	16.0	5.8	0.56	17.4

The best permeation profile was obtained, for both drugs, when we applied them together with Azone in combination with PG: this vehicle was able to increase the clonazepam and lorazepam about threefold compared to the free enhancer formulation (Table 2).

Transcutol proved to be less efficacious, compared to Azone, in promoting the skin permeation of examined benzodiazepines, while limonene, in combination with PG, exerts a moderate enhancing effect in increasing only clonazepam cutaneous flux (EF 2.12; Table 2).

Penetration enhancers may act by altering the diffusion characteristics of the skin or by modifying the SC/vehicle partitioning behavior of the drug. To better understand the enhancement mechanism of used penetration enhancers, we examined the values of some permeation parameters, such as apparent diffusion coefficient (D_{app}) and apparent SC/vehicle partition coefficient (K_{app}), calculated from obtained percutaneous absorption data.

The diffusion coefficient was calculated based on the equation (Flynn et al., 1974):

$$D_{app} = \frac{h^2}{6t_L}, \quad (1)$$

where h is the barrier thickness. Assuming that the SC is the main rate-limiting barrier, h is 16.8 μm for human skin (Bronaugh et al., 1982). The permeability coefficient (P_m) can be evaluated by dividing the steady-state flux (J) by the donor phase concentration (C_{SS}). Therefore, the apparent SC/vehicle partition coefficient can be calculated indirectly from the equation:

$$K_{app} = \frac{P_m h}{D_{app}}. \quad (2)$$

These parameters, as calculated for the permeation of lorazepam and clonazepam, are listed in Table 3.

As shown in Table 3, Azone increased the apparent diffusion coefficient of the drugs but did not affect their apparent SC/vehicle partition coefficient (K_{app}). These findings are in accordance with the suggestion of several authors (Harrison et al., 1996; Bouwstra et al., 1989; Beastall et al., 1988; Goodman and Barry, 1986) that Azone exerts its effect on the drug apparent diffusion coefficient by altering the packing of the bilayer tails within the intercellular space.

Results from our study indicate that Transcutol exerted a moderate effect in increasing skin permeation of both benzodiazepines. Regarding the enhancement mechanism, in our experiments Transcutol increased only the apparent SC/vehicle partition coefficient but did not affect the apparent diffusion coefficient of skin permeation process (Table 3).

This mechanism is consistent with the findings of other authors (Harrison et al., 1996; Panchagnula and Ritschel, 1991) who reported the ability of this compound to promote drug skin absorption by an increased SC/vehicle drug partitioning and an higher drug retention in the skin.

Recently, Mura et al. (2000) have demonstrated the efficacy of Transcutol, alone or in combination with PG, to increase permeability of clonazepam through rabbit ear skin from carbopol gel formulations.

For these authors, the enhancement mechanism involved in the process of clonazepam skin permeation appeared to be related to the solubilizing properties of Transcutol, combined with its ability to increase cutaneous drug retention.

Concerning the enhancement mechanism of limonene, this compound appeared to increase moderately both partition and diffusion apparent coefficients in clonazepam skin permeation process.

Different authors (Okabe et al., 1990) believe that limonene could act as a barrier-altering agent which should allow a reversible change to the skin structure. Cornwell and Barry (1993) assert that D-limonene creates highly permeable micro-pores in the intercellular lipid bilayers through which drugs are able to pass. Other authors (Yamane et al., 1995) reported that limonene in combination with PG can act by a dual mechanism of action: increasing the diffusion coefficient or the partitioning into the skin. This last enhancement mechanism is consistent with the findings obtained in our experiments.

To assess the feasibility of lorazepam and clonazepam transdermal administration in human, blood levels of these drugs following application of transdermal formulations were predicted by using the flux values from the in vitro experiments on excised human skin (Table 2). Steady-state plasma concentration (C_{SS}) can be calculated by means of the following equation (Touitou et al., 1988b):

$$C_{SS} = \frac{JA}{V_d k_e}, \quad (3)$$

where J is the slope of the linear section of the cumulative amount permeated per unit area vs time plot, A denotes the area of application to the skin, V_d (ml) is the volume of distribution, and k_e (h) represents the elimination constant. V_d and k_e of clonazepam and lorazepam in humans are 3000 ml and 2.75×10^{-2}/h and 1500 ml and 4.4×10^{-2}/h, respectively (Clarke, 1986). Thus, for the formulation without penetration enhancers delivering lorazepam or clonazepam and having a flux of 0.48 or 0.32 μg cm^{-2} per h, respectively, blood concentrations of 0.03 and 0.07 μg/ml are predicted for clonazepam and lorazepam using an area of application to the skin of 10 cm^2 for 70 kg human (Table 4). This is only an approximate estimation since biotransformation of both benzodiazepines in their penetration through the excised human skin is not known.

As can be noted from the comparison of theoretical therapeutic concentrations with the experimental data (Table 4), the C_{SS} values were not suitable for transdermal administration of both benzodiazepines. To verify the potential application of enhancer formulations **B** and **G**, which presented the better skin permeation profiles for both benzodiazepines, we calculated the corresponding C_{SS} using the percutaneous fluxes obtained from these vehicles (Table 4). The results obtained suggested that only lorazepam formulation **B** could ensure, after cutaneous application, blood concentrations within the therapeutic range and so the successful transdermal administration of this benzodiazepine.

In conclusion, not all the penetration enhancers tested in this study produced an increase of percutaneous fluxes of clonazepam and lorazepam compared to control formulations. Among the enhancers studied, Azone in combination with PG resulted the most effective in increasing skin permeation of both benzodiazepines. Furthermore, on the basis of a theoretical approach, we can predict lorazepam blood concentrations within the therapeutic range after skin application of **B** formulation. On the basis of these results we

Table 4
Lorazepam and clonazepam plasma concentrations (C_{SS}) and theoretical therapeutic concentrations (C_T) from carbomer gels containing Azone/PG (**B** and **G**) and without penetration enhancer (**A** and **F**).

Gel code	C_{SS} (μg/ml)	C_T (μg/ml)
A	0.07	0.15 ÷ 0.24
F	0.03	0.2 ÷ 0.7
B	0.23	0.15 ÷ 0.24
G	0.11	0.2 ÷ 0.7

believe that lorazepam in association with the enhancers Azone and PG can be regarded as a successful candidate for transdermal administration in humans. However, clinical in vivo studies are needed to validate the feasibility of lorazepam transdermal delivery in humans.

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