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RESEARCH PAPER

Bioadhesive Films Containing Benzocaine: Correlation Between *In Vitro* Permeation and *In Vivo* Local Anesthetic Effect

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ABSTRACT

Purpose The aim of this work was to develop anesthetic bioadhesive films containing benzocaine and study their *in vitro* skin permeation and *in vivo* performance, in comparison with commercial formulations.

Methods Films containing 3% and 5% w/w of benzocaine were prepared and characterized by weight, drug content, thickness and morphology. *In vitro* permeation assays were performed in vertical diffusion cells using full-thickness pig ear skin as barrier. Intensity and duration of analgesia were evaluated in rats by tail-flick test, and skin histological analysis was carried out.

Results Tail-flick test showed that the duration of benzocaine-induced analgesia was significantly prolonged with the films compared to commercial creams, in agreement with the higher *in vitro* permeation. Histological analysis of the rat tail skin did not reveal morphological tissue changes nor cell infiltration signs after application of the commercial creams or films.

Conclusions Results from our study indicate that the films developed in this work can be considered as innovative dermal/transdermal therapeutic systems for benzocaine local delivery.

KEY WORDS benzocaine · *in vitro–in vivo* correlation · local anestetic · patch · transdermal

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INTRODUCTION

Local anesthetics (LAs) are a class of pharmaceutical compounds used to attenuate or to eliminate local pain. Their commercial formulations are used in a variety of routes of administration (injective, topical, dermal and mucosal), for procedures used in medicine and dentistry, on adult and pediatric patients, that require local anesthesia. Local anesthetics can be classified in two classes, amide and ester derivatives (1). Ester-linked LAs are generally shorter acting than amide derivatives, due to the higher susceptibility to enzymatic hydrolysis, but they are also less toxic for the same reason. The most commonly used topical LAs are benzocaine, lidocaine, prilocaine and/or their associations (2–4). Despite the reported cases of methemoglobinemia and allergic contact dermatitis (5,6), benzocaine, an ester-type LA, is used in a wide variety of products such as gels (for toothache), solutions (for otitis), suppositories (for the relief of haemorrhoidal symptoms), creams, ointments and sprays (for skin application). The products destined to be applied on the skin are used in a variety of dermatological procedures and show important clinical results (7). However, the relatively slow absorption and/or fast biotransfor-

mation induces a short duration of local anesthesia restricting its clinical use (2). Additionally, the onset of local analgesia is delayed, and the time for efficacy is typically 30 min to 2 h after drug application (1).

To prolong and improve the local anesthetic effect, new formulations for topical LA application, fast acting and long lasting, would be of extreme interest. Bioadhesion is a possible approach, because it can prolong the contact time between the formulation and the surface to be anesthetized. New topical bioadhesive gels, such as a hydroxypropylmethylcellulose and diethylene glycol-gel (8) and a Carbopol®-liposomal gel (9,10), containing benzocaine have been developed and have shown enhanced local anesthetic effect when applied on the skin. However, the use of semisolid formulations, albeit bioadhesive, presents some disadvantages, because the dose applied and the area of application are unknown and also because the contact time between the formulation and the skin surface is uncontrolled, as the formulation can be removed accidentally. Additionally, the semisolid formulation undergoes a sort of metamorphosis after application, due to the evaporation of volatile components. This leads to a modification of the composition of the formulation applied, which can produce unwanted effects such as drug precipitation/crystallization on the skin surface. The overall result of this metamorphosis is a limited control of drug release. To overcome these problems, a transdermal drug delivery system, in the form of a patch, can be used. Among all trandermal patches available, in this study, we used a new bioadhesive film, recently proposed by some of us (11). The film is water-based, mechanically stable, vapor permeable, and not adhesive in the dry state but only when applied on wet skin, thus reducing the possibilities of skin irritation. This new platform, named Patch-non-Patch®, is a film presenting the adhesive and drug reservoir functions in a monolayer structure (12,13). Recently published studies have reported the applications of the Patch-non-Patch® technology to a variety of actives, such as lidocaine (12,14), caffeine (15), oxybutinyn (16), sumatriptan (17), nicotine and bupropion (18). The results *in vitro* are very promising; however, to date, no *in vivo* efficacy data have been obtained.

Thus, the aim of this work was to develop an enhanced anesthetic bioadhesive film containing benzocaine by studying its *in vitro* skin permeation characteristics and *in vivo* performance, in comparison with commercial formulations. To further improve drug permeation, penetration enhancers, such as Transcutol®, propylene glycol and isopropyl myristate alone or in combination were included in the film. Permeation experiments were performed using pig ear skin as barrier, while tail-flick test on rats was used to assess the analgesic efficacy of selected formulations. The local toxicity of the formulations was determined as well by means of histological analysis. An additional objective of the work was to correlate the *in vitro* permeation kinetics of benzocaine from the studied formulations (commercial creams and prepared films) with the *in vivo* anesthetic/analgesic efficacy.

MATERIALS AND METHODS

Materials

Benzocaine (m.w. 165.19) was a gift from Lisapharma (Erba, I). Polyvinyl alcohol (PVA) of molecular weight 83,000 (degree of hydrolysis 87%) was obtained from Nippon Gohsei (Osaka, Japan). Plastoid® E35H was prepared according to the protocol of Rofarma (Gaggiano, I) (14). Transcutol® was a gift of Gattefossè Italia (Milan, I). Span® 20 and Tween® 80 were obtained from Sigma–Aldrich Co. (St. Louis, USA). All other reagents were of analytical grade.

The commercial formulations Labocaina® (Laleham Healthcare LTD, Alton, UK) and Foille® (Sanofi-Synthelabo, Madrid, E) were purchased in a pharmacy. The composition of Labocaina® (CC-3%, o/w cream) is: benzocaine 3% (w/w), resorcinol 2% (w/w) and chlorotimol 0.032% (w/w) as active agents, water, stearic acid, glycerin, glycerol monostearate, castor oil monostearate, resorcinol, borax, triethanolamine, diethylene glycol monoethyl ether, isopropylic alcohol, zinc oxide and sodium dioctyl sulfosuccinate as inactive ingredients. The composition of Foille® crema (CC-5%, w/o cream) is benzocaine 5% (w/w), benzilic alcohol 4% (w/) and chloroxylenol 0.4% (w/w) as active ingredients, stearic acid, cetyl alcohol, glycerin, liquid paraffin, isopropyl myristate palmitate and stearate, Polisorbate 60, cocoa butter, triethanolamine, Carbomer 974P, sorbitan tristearate, methyl p-idroxybenzoate, eugenol, propyl p-idroxybenzoate, butyl-hydroxy anisole and water as inactive ingredients. The composition of Foille® pomata (CO-5%, w/o ointment) is benzocaine 5% (w/w), benzilic alcohol 4% (w/) and chloroxylenol 0.1% (w/w) as active ingredients, hydrogenated vegetable oil, solid paraffin, yellow beeswax, monoglycerides of fatty acids, corn oil, calcium hydrate, sodium borate, PEG 32, sodium lauryl sulphate, maleic anydhride, eugenol, sodium calcium EDTA, 8-hydroxychinoline, water as inactive ingredients.

Film Preparation and Characterization

Films containing benzocaine were prepared as previously described (11,14). Briefly, a solution of benzocaine in water/plasticizer and cosolvents were added to PVA 25% (w/w) water solution and to the adhesive Plastoid® E35H. As penetration enhancers, Transcutol®, propylene glycol, microemulsion of water, isobutanol, isopropyl myristate, Tween® 80 and Span® 20 (19) or a mixture of ethanol, propylene glycol and Transcutol®, were used and glycerin

as plasticizer. The mixtures obtained were stirred with a magnetic bar overnight, spread on siliconized paper (Bouty S.p.A., Sesto San Giovanni, I) using a film casting knife (BYK Gardner, Silverpring, MD-USA; gap 0.45 mm), oven dried at 80°C during 30 min and then sealed in aluminium pouches. The composition of the wet mass for each formulation is reported in Table I.

The films were characterized by weight, thickness (Absolute Digimatic 547-401, Mitutoyo, Milan, I), morphology (polarized light microscope; Labophot II, Nikon, Tokyo, J) and drug content. For the drug content determination, circles (0.6 cm^2 area) were cut from each film (n=5–6). Each circle was dissolved in 5 mL of NaCl 0.9% solution. After 24 h, the solutions obtained were analyzed by HPLC in order to determine the amount of benzocaine. Drug content was evaluated immediately after preparation and after 3 months of storage at room temperature or at 40°C and 65% of relative humidity. The results were expressed as percentage of benzocaine recovered for each film.

In Vitro Permeation Studies

Permeation assays were performed using vertical Franz-type diffusion cells (Disa, Milan, I) with 0.6 cm^2 permeation area. Full thickness pig ear skin was obtained from a local slaughter-house and served as barrier for all experiments. The skin was excised post-sacrifice from the outer part of pig ears separated from the underlying cartilage with a scalpel and frozen at −20°C until use.

For film application, a known amount of water, namely 15 µl/cm^2, was used (11) prior to film application.

As reference commercial formulations, Labocaina® (CC-3%) and Foille® Crema (CC-5%) containing benzocaine at 3% and 5%, respectively, and Foille® Pomata (CO-5%) were used. All were applied in infinite dose conditions (1 g/cm^2). In a separate set of experiments, Labocaina® (CC-3%) and Foille® Crema (CC-5%) were applied also in finite dose conditions (318 and 510 µg/cm^2 for Labocaina® and Foille®, containing 6.36 mg/cm^2 and 10.2 mg/cm^2 of drug, respectively).

The receptor compartment was filled with 0.9% NaCl solution at 37°C (benzocaine solubility 1.2 mg/ml) under constant magnetic stirring (100 rpm). At predetermined time intervals, aliquots were withdrawn and analyzed by HPLC for drug content determination.

In the experiments made in infinite dose conditions, from the cumulative amounts of benzocaine transported across the skin per unit of area, the flux of drug was calculated from the slope of the linear portion of the curve, typically in the interval 1–8 h. The lag time was obtained from back extrapolation of this straight line to the time axis. The permeability coefficient was calculated according to Eq. 1:

$$J = P \times C_d \quad (1)$$

where J (µgcm^{-2}h^{-1}) is benzocaine flux across the skin, P (cmh^{-1}) permeability coefficient and C_d is benzocaine concentration in the donor (µg/cm^3).

Benzocaine Analysis

Benzocaine analysis was performed by high pressure liquid chromatography (HPLC) using a Perkin Elmer liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) and a UV detector set at 294 nm, according to the method previously described with minor modifications (20). A µBondapak™ C18 analytical column (Waters, Milford, MA, USA) was used. The mobile phase was composed of a mixture of methanol:water (56:40, v/v) containing 4% (v/v) of acetic acid at flow rate of 1.3 ml/min. In these conditions,

Table I Film Composition (% w/w on Wet Mass) and Physical Characteristics

	B01-3%	B02-3%	B03-3%	B04-3%	B05-3%	B05-5%
PVA[a]	56.0	55.0	56.0	56.0	56.0	56.0
Plastoid® E35H	26.0	25.5	26.0	26.0	26.0	26.0
Glycerin	4.0	4.0	4.0	4.0	–	–
Water	13.2	10.7	10.8	9.2	10.8	10.2
Ethanol	–	0.4	–	–	–	–
Transcutol®	–	2.0	2.4	–	2.4	2.4
Propylene glycol	–	1.6	–	–	4.0	4.0
Isopropyl myristate	–	–	–	1.8	–	–
Isobutanol	–	–	–	0.2	–	–
Tween® 80	–	–	–	1.0	–	–
Span® 20	–	–	–	1.0	–	–
Benzocaine	0.8	0.8	0.8	0.8	0.8	1.4
Weight (mg/cm^2)	7.0 ± 0.5	7.5 ± 0.5	4.2 ± 0.4	10.7 ± 0.6	7.8 ± 0.8	7.7 ± 0.3
Thickness (μm)	65 ± 4	67 ± 5	37 ± 3	92 ± 5	68 ± 5	66 ± 3

[a] 25% w/w water solution

benzocaine retention time was 5.5 min. The method was validated according to USP 30.

The stock solution was prepared by dissolving a weighted amount of benzocaine in ethanol:water (50:50 v/v). Five working solutions (which remained stable for 3 days at room temperature) were obtained with appropriate dilution of the stock solution in 0.9% NaCl to give concentrations in the range 0.8–35 μg/ml. For each concentration, three samples were injected to build calibration curves that were analyzed by linear regression analysis of the area of the peak *versus* the concentration. The relative error, which determines the accuracy of the method, resulted in lower than 15%, while relative standard deviation, a measure of precision, was below 1%. The limit of detection resulted in 0.4 μg/ml, and the limit of quantification was 0.8 μg/ml.

Pharmacological and Local Toxicity Assays

Male adults Wistar rats (250–300 g) were obtained from CEMIB-UNICAMP (Centro de Bioterismo, State University of Campinas—UNICAMP, Campinas, São Paulo, Br). Protocols were approved by the UNICAMP Institutional Animal Care and Use Committee, which follows the recommendations of the Guide for the Care and Use of Laboratory Animals (protocol number 1551-1).

For the pharmacological evaluation, the formulations were selected according to the *in vitro* permeation results. The animals, randomly selected for the pharmacological assay, were divided in groups of 5–7 each and treated by topical application with 12.7 mg of CC-3% or 20.4 mg of CC-5%, spread over an area of 2 cm^2 and with 2 cm^2 films on the dorsal surface of the tail 5 cm over the root. Placebo film B03 and B04 were used as control groups.

Tail-Flick Test

The analgesic effects evoked by benzocaine commercial creams and films were evaluated using the tail-flick test. First, the animal was placed in a horizontal acrylic restraint and fixed on an analgesimeter with a portion of the tail, 5 cm from its tip, exposed to heat from a projector lamp (55 ± 1°C). A single control switch simultaneously activated the light and a timer. The timer stopped when the exposed rat tail flicks, and the interval between switching on the light and flick of the tail was recorded (latency time). A 30-s cut-off time was used to avoid thermal injury, and the baseline (normal response to the noxious stimulus) was recorded before starting the experiments. The evaluation was started 30 min after application of the formulations and the data recorded at time intervals of 30 min during the firsts 2 h and of 60 min up to 6 h after treatment. Data, expressed as percent of animals with analgesia, duration of the analgesic effect (minutes) and area under the efficacy curve (AUEC) were recorded for each experimental group.

Histological Assays

Animals were sacrificed under anesthesia (urethane 1 g/kg and alfa-chloralose 50 mg/kg) 24 h after treatment. In order to evaluate the surroundings of the site of application, the tail skin was dissected out, fixed with Bowin solution (750 ml picric acid saturated aqueous solution, 250 ml 37–40% formalin and 50 ml glacial acetic acid) (21) during 24 h and, after that, with 10% formalin solution for 48 h. Skins were embedded in paraffin, and five transverse sections (6 μm of thickness) were obtained. The samples were stained with hematoxylin and eosin (H&E) and analyzed using light microscopy. Low-power video images (10 × objective) of the entire cross-section were taken with a highly sensitive video camera (Sony CCD) linked to a light microscopy and enhanced with an image processor system (CoolSnap, Media Cybernetics, USA). Possible structure changes and cell infiltration were evaluated by an analyzer blinded to the treatments.

Statistical Analysis

Characterization, *in vitro* permeation and *in vivo* pharmacological data were expressed as percentage or mean±sd and analyzed by one-way analysis of variance (one-way ANOVA) with Tukey-Kramer or Bonferroni *post hoc* tests using Graph Pad Instat (Graph Pad Software Inc., USA) or Origin 6.0 (Microcal™ Software, Inc., Northampton, MA, USA) programs. Statistical differences were defined as $p<0.05$ by One-way ANOVA (22).

RESULTS AND DISCUSSION

In this study, we prepared and tested *in vitro* and *in vivo* bioadhesive films containing benzocaine with or without penetration enhancers. Using this delivery platform, for most of the drug studied, the permeation kinetics of model compounds across the skin were not linear, but depended on the square root of time, suggesting that the film acts as a matrix controlling the release of the drug [12]. This leads to a release rate of the drug that is particularly high at short application times, with the possibility to achieve a faster onset of action *in vivo* compared to the commercial formulations.

The literature reports that benzocaine can be metabolized during its transport across viable skin *in vitro* (23): the main metabolic pathway is N-acetylation, which forms N-acetyl benzocaine, while hydrolysis, forming para-amino benzoic acid, accounts only for 10% of the dose applied (23). In our *in vitro* permeation experiments, skin metabolism was not detected, probably because the skin used was

previously frozen and thus non-viable. On the other hand, N-acetylation, the prevalent metabolic pathway in viable skin, leads to a derivative, which has the same biological activity of benzocaine.

Film Characterization and Stability

Six films containing benzocaine were prepared (see Table I). The first film produced (B01-3%), was composed only of a film-forming agent (PVA), an adhesive (Plastoid® E35H) and a plasticizer (glycerin). In order to increase benzocaine permeation, Transcutol® (B03-3%), a mixture of Transcutol®, ethanol and propylene glycol (B02-3%), isopropyl myristate, Tween® 80 and Span® 20 (B04-3%), and mixture of Transcutol® and propylene glycol (B05-3%) were incorporated as permeation enhancers. Film B05 was prepared either with a drug content of 3% or of 5% (w/w) on finished product.

All films were characterized by weight, thickness, drug content and morphology (see Table I and II). The weight of the films obtained ranged from 4.2 ± 0.4 mg/cm^2 for film B03-3% to 10.7 ± 0.6 mg/cm^2 for film B04-3%, and the thickness from 37 ± 3 μm of film B03-3% to 92 ± 5 μm for film B04-3%. The differences in thickness and weight per unit area are simply due to the presence in the heaviest and thicker films of a higher proportion of non-volatile components.

The analysis of films surface with a polarized light microscope revealed the presence of benzocaine crystals in the case of formulations B01-3%, B02-3% and B03-3%, shortly after their preparation. No benzocaine crystals were observed for film B04-3% and B05, both for 3% and 5% drug content, probably because of the higher solubility of benzocaine into the film, due to the presence of isopropyl myristate and of the mixture of Transcutol® and propylene glycol.

The drug content, expressed as μg/cm^2 and percentage of benzocaine on finished product, is reported in Table II. Benzocaine content did not change after 3 months of storage at either room temperature or at 40°C and 65% RH, suggesting that all films are stable in these conditions.

In Vitro Permeation Studies

Fig. 1 illustrates the permeation profiles of benzocaine across pig ear skin from three commercial formulations containing benzocaine applied in infinite dose conditions. Two formulations were creams, containing either 3% (CC-3%) or 5% (CC-5%) of benzocaine, while one, CO-5%, was a 5% ointment. The 3% cream was an o/w formulation, while the 5% cream was a w/o emulsion. Surprisingly, despite the different drug contents and the composition of the three formulations, the permeation profiles were practically superimposed.

The permeation parameters, namely flux, permeability coefficient and lag-time, were calculated according to Eq. 1, and the respective values are reported in Table III. Flux values were comparable for the three formulations (in the range from 44.7 to 47.2 μg cm^{-2}h^{-1}), whereas the permeability coefficient was, as expected, significantly higher for the 3% cream. The reason for this behavior is not known, but is probably linked to the different composition and nature of the formulations. Formulations containing the same drug but different excipients can produce difference in drug transport across a membrane such as the skin, owing to differences in drug solubility and partitioning. The driving force in transdermal drug diffusion is the thermodynamic activity of the permeant in the vehicle, which is typically approximated to concentration, even though this approximation is valid only in dilute solution. It has been shown that drug permeation across a membrane can be comparable even from different vehicles and different drug concentrations, provided that the thermodynamic activity of the drug in the vehicles is the same. So, the three formulations used in the present work are different in terms of drug concentration and excipient composition, but they have evidently the same thermodynamic activity.

Due to the similarity in performance of the ointment CO-5% and cream CC-5%, the ointment was not used in the following tests, which were performed only on creams (CC-3% and CC-5%). Initially, the two creams were tested

Table II Benzocaine Content in the Dried Films and Stability Data ($n = 4$–6)

Formulations	Benzocaine content			
	T0		% (w/w) after 3months	
	μg/cm^2	% (w/w)	25°C	40°C
B01-3%	186.0 ± 14.4	2.48 ± 0.09	2.56 ± 0.09	2.66 ± 0.14
B02-3%	194.8 ± 13.4	2.70 ± 0.05	2.73 ± 0.02	2.84 ± 0.03
B03-3%	101.7 ± 14.0	2.50 ± 0.04	2.66 ± 0.27	2.44 ± 0.09
B04-3%	310.2 ± 11.2	2.80 ± 0.05	2.97 ± 0.02	3.06 ± 0.03
B05-3%	220.3 ± 29.4	2.80 ± 0.13	2.66 ± 0.27	2.73 ± 0.01
B05-5%	374.8 ± 24.4	4.68 ± 0.34	4.33 ± 0.26	4.65 ± 0.14

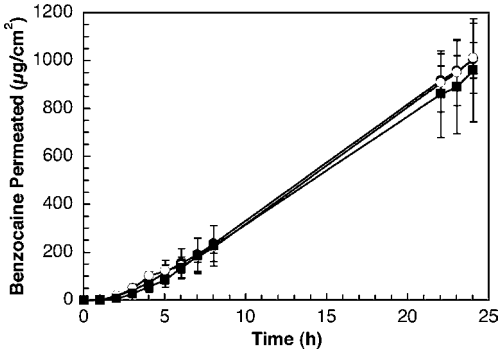

Fig. 1 Permeation profiles of benzocaine across pig ear skin from commercial formulations applied in infinite dose conditions (mean values±sd). (*Filled circle*) Commercial cream containing 3% (w/w) of benzocaine (CC-3%), (*White circle*) Commercial cream containing 5% (w/w) of benzocaine (CC-5%), (*Filled square*) Commercial ointment containing 5% (w/w) of benzocaine (CO-5%).

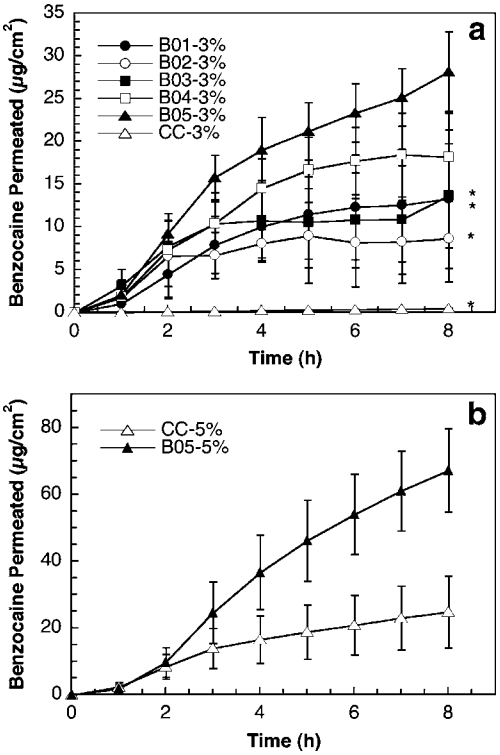

Fig. 2 Permeation profiles of benzocaine from commercial formulations applied in finite dose conditions and from films (mean values±sd). Panel (**a**) reports the data obtained from formulations containing 3% of benzocaine, Panel (**b**) reports the data obtained from formulations containing 5% of benzocaine. *significantly different from B05-3% ($p < 0.05$).

at finite dose to simulate the conditions of application *in vivo*. Fig. 2 reports the permeation profiles obtained: Panel a refers to the 3% formulation (CC-3%, dose applied 6.4 mg/cm^2) while Panel b is relative to the 5% formulation (CC-5% dose applied 10.2 mg/cm^2). The permeation of benzocaine across the skin was, as expected, much lower in finite dose conditions compared to the infinite dose conditions, but a difference emerged between the two commercial creams; in particular, CC-3% gave a much lower permeation compared to CC-5% cream, not justified by the difference of drug concentration alone. This can be appreciated better by looking at the percentage of benzocaine permeated after 8 h, which is reported in Fig. 3: it emerges that from CC-3% the percentage permeated was 0.15±0.01, while it reached 4.88±2.11 in the case of CC-5%. The difference was probably due to the different excipients contained, which leads to a different metamorphosis of the two creams when applied. In fact, the CC-3% cream is a o/w emulsion, containing also solid components such as zinc oxide: when applied on the skin surface, it dries out quite quickly, leaving a paste-like residue, across which drug diffusion was probably not favored. The CC-5% formulation is a w/o cream, relatively unctuous, which did not change to a significant extent on the skin surface, leading to a higher release of the drug. The difference between the two formulations was not evident in infinite dose conditions (see Fig. 1), because both creams remained unchanged until the end of the experiment, as no solvent evaporation took place.

Besides commercial creams the prepared films were also tested. The first film, B01-3%, did not contain any enhancers; however, it gave rise to a permeation profile that was higher than that of the commercial formulation CC-3%. The profile tended to flatten after 4 h of application, indicating a decreasing permeation rate with time. A similar permeation profile has been already observed with other actives, especially sumatriptan (17), and has been attributed

Table III Permeation Parameters of Benzocaine Across Pig Ear Skin from Commercial Formulations Applied in Infinite Dose Conditions. Mean Values ±SD

Formulation	Emulsion type	Flux (μgcm^{-2}h^{-1})	Permeability coefficient (cmh^{-1})×10^3	Lag time (h)
CC-3%	o/w	47.2±5.1	1.57±0.17[a,b]	2.6±1.4
CC-5%	w/o	45.4±7.2	0.93±0.14[a]	2.4±0.4
CO-5%	w/o	44.7±9.9	0.89±0.20[b]	2.7±0.8

[a] significantly different ($p < 0.01$)

[b] significantly different ($p < 0.05$)

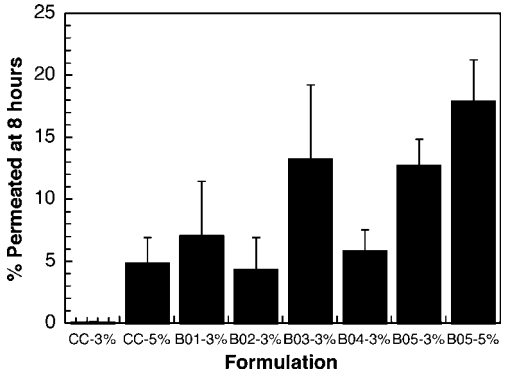

Fig. 3 Percentage of benzocaine permeated after 8 h from all formulations tested in finite dose conditions (mean values±sd).

to local drug depletion in the portion of the film in contact with the skin and to the difficult diffusion of the drug through the dry polymeric matrix.

The inclusion of Transcutol® alone (B03-3%) or together with propylene glycol (B02-3%) did not modify the permeation profile to a significant extent. When isopropyl myristate, Tween® 80 and Span® 20 were added (B04-3%), the permeation profile increased slightly compared to the formulation without enhancers. When Transcutol® and propylene glycol were used at higher concentration (B05-3%) the amount of benzocaine permeated increased in a significant way compared to B01-3%, B02-3% and B03-3%. Concerning the formulation containing 5% of benzocaine, B05-5%, the film had a better performance compared to the commercial cream CC-5% (Fig. 2, Panel b).

Microscopic analysis of the films provided information about the differences observed, showing that benzocaine crystals were present on the surface of B01-3%, B02-3% and B03-3% films (as observed by polarized light microscopy, data not shown). On the contrary, when isopropyl myristate, Tween® 80 and Span® 20 (B04-3%) or Transcutol® and propylene glycol at higher concentration (B05 films) were present, no crystals were observed, indicating that these solvents at the concentration used were able to solubilize completely the amount of benzocaine present. The association Transcutol® plus propylene glycol gave a more pronounced enhancement compared to isopropyl myristate, Tween® 80 and Span® 20. Transcutol®, a powerful solubilizing agent, is known to increase the solubility of both lipophilic and hydrophilic permeants into the stratum corneum (24), thus acting as penetration enhancer. When used in combination with propylene glycol, a further increase in skin penetration was observed for clonazepam (25). Then, the inclusion of Transcutol® and propylene glycol, at a certain concentration, was able not only to completely solubilize benzocaine but also to act as penetration enhancer.

Because the amount of benzocaine applied (expressed as $\mu g/cm^2$) was different for the different films (see Table III), the cumulative amount of benzocaine permeated at 8 h was normalized by the amount applied, and the resulting percentage permeated is reported in Fig. 3. The results obtained indicate that the use of Transcutol®/propylene glycol at low concentration (B02-3%) or isopropyl myristate (B04-3%) did not exert any penetration enhancer effect when incorporated into the film, because the percentage permeated was the same as with the "plain" film (B01-3%). The higher percentage of benzocaine permeated from the B05-3% and B05-5% films is presumably due to the solubilizing and enhancing effects of Transcutol® and propylene glycol.

Pharmacological and Local Toxicity Evaluation

The films B03-3%, B05-3% and B05-5% were selected for the pharmacological assays considering the high percentage of drug permeated, as previously described in percutaneous permeation studies.

Fig. 4 illustrates the analgesic efficacy of the benzocaine commercial creams and different films at 3 and 5%. The area under the efficacy curve of the rat-tail-flick test ($AUEC_{0-360}$) showed an increase in analgesic activity of benzocaine after the treatment with the films B03-3% ($AUEC_{0-360}$=10,249), B05-3% ($AUEC_{0-360}$=18,249) and B05-5% ($AUEC_{0-360}$=

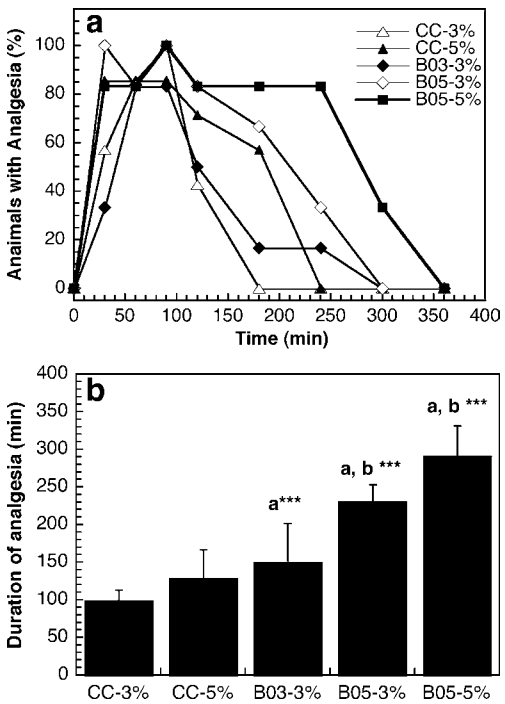

Fig. 4 Time-course (min) showing the percent of animals with analgesia (Panel **a**) and the duration of analgesia (Panel **b**) evaluated by the tail-flick test in rats after treatment with commercial creams and films containing benzocaine (n=5–7/group). Data expressed as mean±sd ***$p<0.001$. One-way analysis of variance with Bonferroni *post-hoc* test. **a** B03-3% and B05-3% *vs.* CC 3%; **b** B05-3% or B05-5% *vs.* CC-5%.

23,749) application compared to the CC-3% ($AUEC_{0–360}$ = 9,210) and CC-5% ($AUEC_{0–360}$ = 14,356), indicating the enhanced local anesthetic effects of benzocaine.

Additionally, as can be seen in panel b, the time course of tail-flick test showed that the duration of benzocaine-induced local anesthetic effect was prolonged after application of B03-3%, B05-3% and B05-5%, showing significant statistical differences in relation to the 3% ($p < 0.001$) and 5% ($p < 0.001$) commercial creams. As expected, no signs of analgesic effects were observed in the animals treated with placebo films containing Transcutol® or Transcutol®/propylene glycol (data not shown).

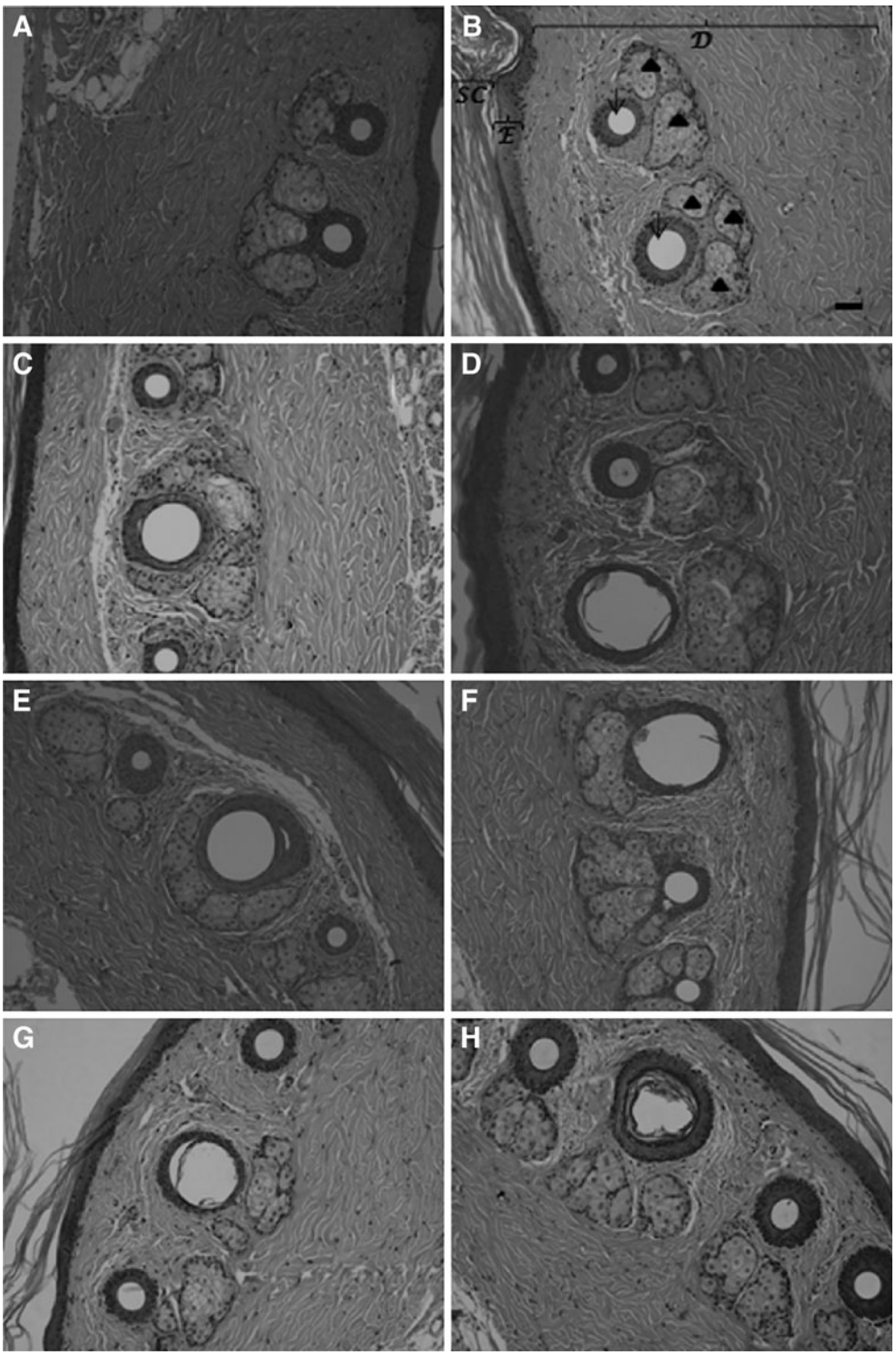

Fig. 5 Transverse sections of rat tail skin showing no treated group (**a**); placebo films containing only Transcutol® (PL-T) (**b**) or Transcutol® -propylene glycol (PL-T/P) (**c**); 3 and 5% commercial creams (CC-3% and CC-5%) (**d** and **e**); 3% benzocaine film formulation 03 (B03-3%) (**f**); 3% benzocaine film formulation 05 (B05-3%) (**g**) and 5% benzocaine film formulation 05 (B05-5%) (H) ($n = 5–7$/group). SC: stratum corneum; E: epidermis; D: dermis. Sebaceous gland (*arrow head*) and hair follicle (*arrow*) are also observed. No morphological tissue changes were detected after treatment with all formulations. *Scale bar*: 20 μm.

Morfological changes, such as epidermal liquefaction, oedema of collagen fibres and also cell infiltration, were the main parameters to evaluate the effects of drug and their permeation enhancers (26). Histological analysis of the rat tail skin did not reveal morphological tissue changes nor cell infiltration signs after application of the commercial creams or films, since the structure of the stratum corneum, epidermis and dermis were preserved, as observed in Fig. 5. In this manner, it was possible to achieve enhanced and prolonged analgesic effects without superficial skin irritation nor morphological changes on skin tissue, even in the presence of penetration enhancers such as propylene glycol and Transcutol®.

Comparison of *In Vitro* and *In Vivo* Results

It is well known that rodent skin is more permeable (due to the differences in thickness, composition and structure) than human and pig skin (27,28). Despite the well-described *in vitro* percutaneous studies across pig skin, the evaluation methods and the behavior response for analgesic effects in pigs are not well established. For this reason, the pharmacological evaluation studies are performed using a rodent model.

As a general rule, the analgesic response to benzocaine should be related to the amount of drug that reaches the target site, i.e. the nociceptors located in the dermis. The local concentration of drugs in the dermis is not easy to determine, and the available techniques, such as microdialysis or drug extraction upon separation from epidermis, are complex, time consuming and still need substantial further development and validation (29). For these reasons, benzocaine dermis concentration was not determined, but drug flux was determined instead. On the other hand, it has been shown that drug concentration into the stratum corneum, the main barrier to drug transport across the skin, is related to the amount penetrated across the skin (30), so benzocaine flux can be taken as a rough estimate of the amount of drug reaching the target site.

In Fig. 6, the analgesic response (duration of analgesia and $AUEC_{0–360}$) obtained *in vivo* is reported against the cumulative amount of benzocaine permeated after 6 h *in vitro*. Both the duration of analgesia and the $AUEC_{0–360}$ increase with the amount of benzocaine permeated across the skin *in vitro* (regression equations are: $y = 102.17 + 3.54x$, $R^2 = 0.82$ for duration and $y = 8901.43 + 285.21x$, $R^2 = 0.92$, $AUEC_{0–360}$).

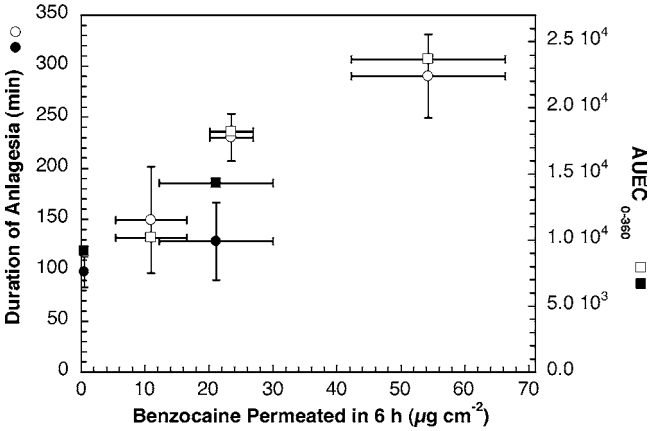

Fig. 6 Relationship between the cumulative amount of benzocaine permeated across the skin at 6 h and the $AUEC_{0–360}$ obtained. *Circles* refer to duration of analgesia, while *squares* refer to $AUEC_{0–360}$. *Full symbols* refer to commercial formulations, *empty symbols* to the films tested.

CONCLUSIONS

In this study, we have successfully developed bioadhesive films containing either 3% or 5% of benzocaine to be applied on the skin surface to achieve local anesthetic effect. The prepared films showed analgesic effects against physical stimulus-induced pain in rats, and they were more effective than commercial semisolid formulations with the same drug concentration, in agreement with the higher *in vitro* permeation. Among the penetration enhancers studied, such as Transcutol®, propylene glycol and isopropyl myristate used alone or in combination, only Transcutol® plus propylene glycol at appropriate concentration (8 and 14% w/w on dry basis, respectively) resulted in effectively promoting benzocaine transport and analgesic effect, compared to the control film. Histological analysis of the rat tail skin did not reveal morphological tissue changes nor cell infiltration signs after application of the commercial creams or films.

Overall, the results from our study indicate that the films containing appropriate amounts of the penetration enhancers propylene glycol and Transcutol® can be considered as innovative dermal/transdermal therapeutic systems for benzocaine delivery, since they are fast acting and long lasting, without local toxicity. This feature, prolonged analgesia with short latency, has an enormous clinical relevance.

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