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ORIGINAL ARTICLE

Transdermal penetration of diclofenac in the presence of AAPH-derived peroxyl radicals

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Abstract

In vitro transdermal delivery of diclofenac across pig skin in the presence of AAPH-derived peroxyl radicals (2,2′-azo-bis(2-amidinopropane)dihydrochloride) has been studied. The transdermal absorption of diclofenac was estimated using high-performance liquid chromatography after its incubation in vertical cells. The diclofenac transdermal penetration was highly modified by peroxyl radicals. In the presence of AAPH, lower diclofenac absorption was observed. This effect was inhibited by ferulic acid. This could be explained by diclofenac–peroxyl radical interactions. In the pretreatment skin with AAPH, lower diclofenac absorption was higher than control experiments. This behavior should be considered in the topical administration of pharmaceutical preparations containing diclofenac.

Key words: *AAPH; absorption; diclofenac; peroxyl radicals; transdermal*

Introduction

Diclofenac is a highly effective nonsteroidal anti-inflammatory agent (NSAID) widely employed in the treatment of inflammation, pain, and musculoskeletal disorders. Its mechanism of action is related to the inhibition of the cyclo-oxygenase enzyme[1]. There are different pharmaceutical dose forms, such as injections, orally sustained release tablets, and topical formulations. In recent years, it has been suggested that topically-applied diclofenac is a good alternative for the treatment of rheumatic diseases, with similar efficiency to other oral NSAIDs[2]. In spite of the well-known anti-inflammatory properties of this drug, some free radical scavenging ability has been reported. Diclofenac has been shown to inhibit the oxidation of phosphatidylcholine liposomes[3] and linoleic acid[4]. In addition, it has been shown to reduce the production of phosphatidylcholine hydroperoxide in rat plasma subjected to the ischemia-reperfusion process[5]. Furthermore, You-Zhi and Zai-Qun[6] have reported that diclofenac protects human erythrocytes against the hemolysis process induced by peroxyl radicals.

Furthermore, it has been demonstrated that human skin is a target of oxidative stress generated from UV radiation or chemical (xenobiotic) agents. In addition, environmental pollutants could directly or indirectly produce reactive oxygen species (ROS)[7].

To our knowledge, works have not been developed aimed at studying the effect of peroxyl radicals on the absorption of topical diclofenac formulations. Thus, the objective of this article was to evaluate the transdermal penetration of diclofenac in the presence of AAPH-derived peroxyl radicals.

Materials and methods

Reagents

Diclofenac sodium salt was purchased from Instituto Sanitas Labotarory (Chile); 2.2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH) and ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (high performance liquid chromatography [HPLC] quality) was from Merck S. A. (Germany). All

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other chemical reagents (sodium acetate, Hepes, etc.) were of analytical grade.

Preparation of Skin

Skin was obtained from various regions (neck, hind leg, fore leg, back) of a 2- to 3-day-old pig[8–10]. The skin was frozen (for no more than 3 months) at −20°C until use. About 1 hour before each experiment, the skin was thawed and any hair was cut short. The skin was then cut into appropriately sized pieces, which were placed in the diffusion cells with the epidermis facing the donor solution.

Transdermal diffusion experiments

Vertical diffusion cells of 4.15 cm^2 were used (Laboratory Glass Apparatus Inc., Miami, FL, USA), thermostatized at 37°C. Heppes buffer (25 mM, pH 7.4, 5 mL) was used as receptor solution. A donor solution containing diclofenac (1–15 mM) with or without AAPH (10 mM) in Heppes buffer (25 mM, pH 7.4, 3 mL) was employed. In some experiments, ferulic acid (50 μM) was added to donor solution. After 5, 10, and 24 hours of incubation, the concentration of diclofenac in the receptor solution was quantified. At each time, all of the receptor solution was sampled and the receptor chamber refilled with fresh buffer. All experiments were performed with five or six replicates.

In some experiments, the skin was pretreated with AAPH. Pig skin was incubated at 37°C for 12 hours in the presence of AAPH 10 mM. After this preincubation, the solution containing AAPH (the donor solution) was removed and replaced with a diclofenac 1 mM solution (in Hepes 25 mM, pH 7.4).

Diclofenac quantification

Quantification of diclofenac in receptor solutions was as follows: First, all of the receptor solution was evaporated to dryness in an oven (Fisher Isotemp Oven Senior Model) at 50°C. The dry residue was then dissolved in 0.5 mL of the HPLC mobile phase (see below), and the solution was then centrifuged (Universal 32R Hettich Zentrifugen) for 15 minutes at 11,000 rpm. The supernatant was filtered (Sartorious AG-37070, Germany, 0.45 μm), and the drug was quantified in the filtrate by HPLC as below.

Diclofenac was quantified by HPLC, using a Shimadzu C-R6A HPLC with a Shimadzu LC-9A pump, a Shimadzu SPD-6A detector, and a Shimadzu C-R6A chromatopac (SP4290). The mobile phase was acetonitrile/buffer acetate (0.2 M, pH 6.3) 40:60, degassed by vacuum filtration. The column was a Lichrospher® 100 RP-18 5 μm (Merck S. A.) and the precolumn was a Lichrospher® 100 RP-18 5 μm (Merck S. A.). The flow rate was 1 mL/min and the diclofenac was quantified on the basis of absorption at 280 nm. The sample volume was 20 μL. The calibrated line equation obtained was y (μg/mL) = 108,351 + 15,714 area (R = 0.999, n = 6).

Data analysis

At least five or six replicates of each experiment were used. Results are presented in the text as mean + *SD*s. Data was analyzed by variance analysis, and Dunn's tests or Student–Newman–Keuls tests for comparisons of multiple means. Statistical significance was fixed at $P < 0.05$[11,12].

Results and discussion

Influence of peroxyl radicals in the transdermal penetration of diclofenac

The transdermal absorption of diclofenac was studied in both the absence and presence of AAPH. As can be seen in Figure 1, in the absence of AAPH, the diclofenac transdermal penetration was dependent on its concentration in the donor solution. Rhe highest absorption penetration was observed for diclofenac 15 mM. In addition, diclofenac transdermal penetration was dependent on incubation time. For example, the transdermal penetration was dependent on incubation time. For example, the transdermal absorption of diclofenac at 1 mM concentration was linearly dependent with a line equation: y (μg/cm^2) = −1.347 + 0.67964 h, r = 0.98672.

The reported water solubility of diclofenac is close to 9 mg/mL (30 mM)[13]. However, in solutions containing

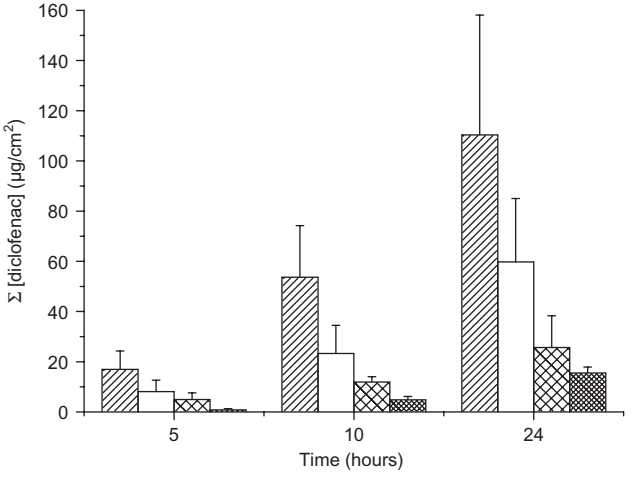

Figure 1. Effect of the drug concentration on the transdermal delivery of diclofenac (mean ± SD, n = 5–6). 15 mM, 7 mM, 3 mM, 1 mM.

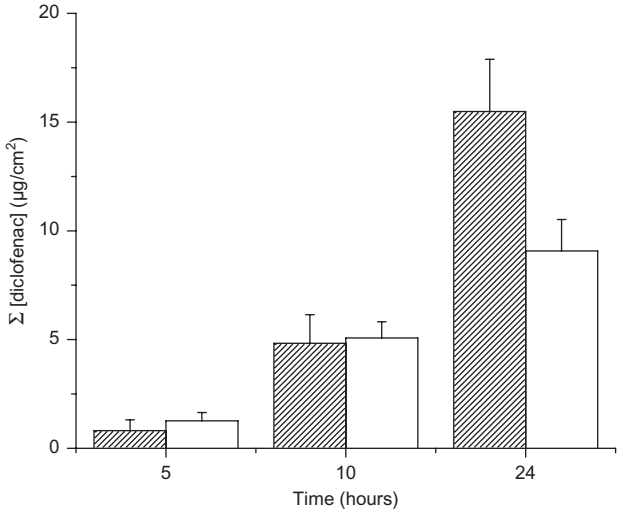

Figure 2. Influence of AAPH on the transdermal penetration of diclofenac. Passive diffusion of diclofenac without AAPH (control experiments). ▨, Passive diffusion of diclofenac with AAPH □ (mean ± SD, n = 5–6) (diclofenac 1 mM, AAPH 10 mM).

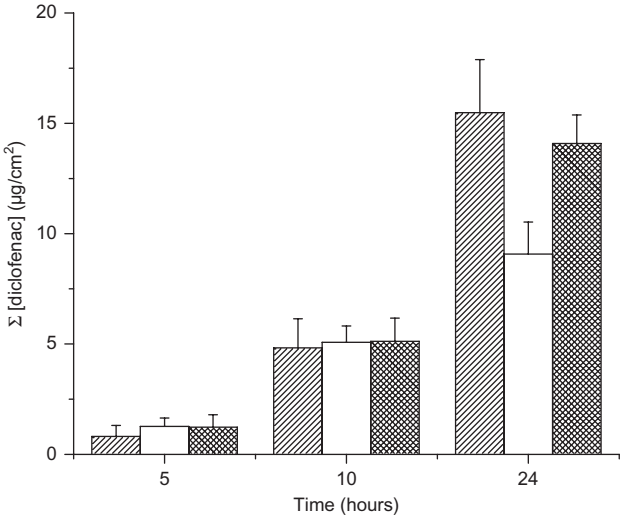

Figure 3. Influence of the antioxidant ferulic acid on the transdermal penetration of diclofenac in the presence of peroxyl radicals. Passive diffusion of diclofenac (control experiments) ▨, passive diffusion of diclofenac with AAPH. □, Passive diffusion of diclofenac with AAPH + ferulic acid ▩ (mean ± SD, n = 5–6) (diclofenac 1 mM, AAPH 10 mM, ferulic acid 50 μM).

AAPH (10 mM) and diclofenac concentrations higher than 5 mM, a precipitate was observed. Therefore, for AAPH-skin assays, a 1 mM diclofenac concentration was used. In these experiments, AAPH (10 mM final concentration) was added at 10 hours of incubation. Figure 2 shows the effect of AAPH addition on diclofenac transdermal absorption. As can be seen in this figure, the absorption of diclofenac in the absence of AAPH was 1, 5, and 15 μg/cm^2 at 5, 10, and 24 hours, respectively. The addition of AAPH strongly modified the drug penetration with only 9.1 μg/cm^2 of diclofenac absorbed after 24 hours. This result is statistically different than the control experiment and implies that diclofenac absorption was reduced by 40%. These results (Figure 2) could be explained in terms of a diclofenac–peroxyl radicals interaction. In this context, different reports have been published suggesting that diclofenac is able to react with free radicals[6,14]. Therefore, the lower absorption of diclofenac in the presence of AAPH could be related to an interaction of diclofenac with AAPH-derived peroxyl radicals, and/or to reactions involved in the lipoperoxidation processes. In both cases, diclofenac would provide protection to the skin against lipid peroxidation caused by peroxyl radicals damage[3–5,15].

To test whether the lower penetration of diclofenac in the presence of AAPH is due to an interaction of diclofenac with free radicals, some experiments with ferulic acid were carried out. Ferulic acid has shown to be a good antioxidant able to protect target molecules and cells from the oxidation processes induced by AAPH-derived peroxyl radicals[16–20]. Moreover, ferulic acid has been proposed as a topical protective agent against UV radiation-induced skin damage[21].

As is shown in Figure 3, the addition of AAPH (10 mM) and ferulic acid (50 μM) after 10 hours of incubation modified the diclofenac transdermal penetration. After 24 hours of incubation, the diclofenac transdermal absorption was 15 μg/cm^2 in the absence of AAPH and ferulic acid (control experiments), 9.1 μg/cm^2 in the presence of AAPH and 14.5 μg/cm^2 in the presence of both AAPH and ferulic acid. These experiments showed statically significant differences among all them.

These results imply that ferulic acid inhibited efficiently the interaction of diclofenac with peroxyl radicals. This agrees with the lower scavenging activity of diclofenac reported by Fodor et al.[14] and with the antioxidant efficiency reported for ferulic acid[19]. The addition of ferulic acid suggests that the changes observed in the transdermal penetration of diclofenac in the presence of AAPH could be related to free radical processes. Taking into account that diclofenac is a mild peroxyl radical scavenger and that the free radicals could initiate some damage to the skin, experiments in skin preincubated with AAPH were developed.

Effect of skin preincubation with AAPH in diclofenac transdermal absorption

To estimate the effect of peroxyl radicals on skin, pig skin was preincubated with AAPH (10 mM) for 12 hours before the diclofenac experiments. After skin incubation, the AAPH solution was replaced with the diclofenac solution. Figure 4 shows the penetration of diclofenac in the pretreated skin. Interestingly, after

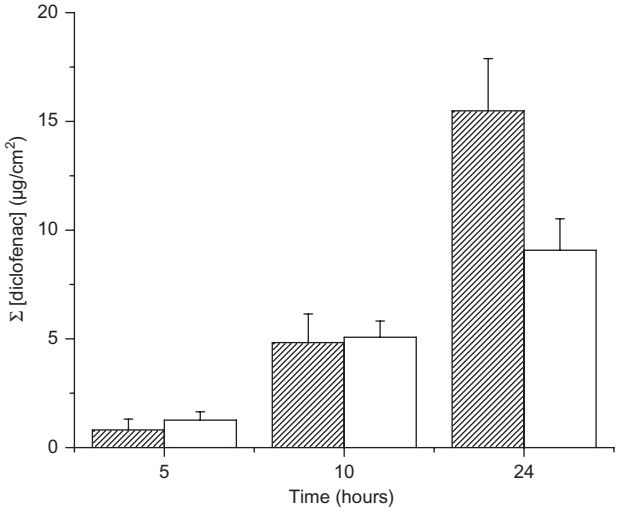

Figure 4. Influence of the interaction AAPH-skin on the transdermal penetration of diclofenac. Passive diffusion of diclofenac (control experiments). ▨, Passive diffusion of diclofenac after preincubation with AAPH □ (mean ± SD, n = 5–6) (diclofenac 1 mM).

24 hours of diclofenac incubation, a higher absorption of diclofenac was observed. Thus, while 15 μg/cm^2 of diclofenac penetrated 'normal' skin, 67 μg/cm^2 was quantified in the previously treated skin (statically significant differences). This result implies that the effect of diclofenac is strongly dependent on the skin's oxidative stress. When diclofenac is present during lipoperoxidation processes, lower diclofenac absorption was observed. In contrast, when skin was previously incubated with AAPH-derived peroxyl radicals, the absorption of diclofenac was higher than both AAPH and control experiments.

Conclusions

Diclofenac transdermal penetration was modified by peroxyl radicals. This effect was explained by both reactions of diclofenac with free radical and changes in the skin structure. These properties should be considered in the topical administration of pharmaceutical preparations containing diclofenac.

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Declaration of interest: The authors report no conflicts of interest.

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