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Original scientific paper

Permeation of Indomethacin through Skin Using Nanonized Alaptide

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Abstract

Alaptide, (S)-8-methyl-6,9-diazaspiro[4.5]decan-7,10-dione, is an original Czech compound; in this paper it is used as an excipient. The investigation deals with the affection of the permeation of indomethacin through full-thickness pig ear skin using a Franz diffusion cell from the donor vehicle of propylene glycol/water (1:1) using nanonized alaptide as a potential transdermal permeation enhancer. Alaptide was applied in ratio 1:10 (w/w) related to the amount of indomethacin. Nanonized alaptide showed an excellent rapid onset of enhancement effect, already at the 30th minute after application, when the permeated amount of indomethacin was 5-fold more than in the formulation without alaptide. The enhancement ratio of nanonized alaptide was 5.6, which indicates that alaptide modifies skin structure, which results in significantly enhanced permeation at long-term application.

Keywords

Alaptide; indomethacin; nanoparticles; permeation; skin.

Introduction

Transdermal administration of drugs can be considered as an alternative to conventional pharmaceutical dosage forms. Nevertheless, a frequent problem of transdermal drug delivery is insufficient or no penetration of active pharmaceutical substances through the skin, and thus various approaches to overcoming the skin barrier was to be developed. These approaches can be based on an optimization of a drug/vehicle system or modification of *stratum corneum* (which is the outermost layer of the skin responsible for barrier function and formed by corneocytes and an intercellular lipid matrix). Modification (i.e. hydration, lipid fluidization and/or disruption) of *stratum corneum* is possible through: (i) application of transdermal chemical permeation enhancers (CPEs); (ii) overall optimization of formulation using non-hydrophobic excipients; (iii) application of physical enhancement techniques (electrically assisted methods), such as iontophoresis, electroporation, acoustic methods, microneedles, etc.) [1–3 and refs. therein].

CPEs can be considered as excipients specifically affecting intercellular space between corneocytes or modifying corneocytes by hydration or denaturation of keratin. The heterogeneity of molecular structures of CPEs limits simple explanation of their action. Several possible mechanisms of action were hypothesized, but exact mechanisms have not been elucidated. It is almost certain that CPEs exhibit multiple effects:

(i) interact with the intercellular lipid matrix; (ii) interact with proteins (influencing the conformation of keratin in corneocytes or proteins in desmosomes); (iii) promote partitioning (influencing the *stratum corneum* nature leads to raising the penetrant concentration gradient and thus increasing the flux, i.e. increasing the concentration of the drug in the skin) [1–3 and refs. therein].

Alaptide, (*S*)-8-methyl-6,9-diazaspiro[4.5]decan-7,10-dione, (Figure 1) is an original Czech compound prepared by Kasafirek *et al.* in the 80s of the 20th century [4]. A class of similar compounds was designed as analogues of melanocyte-stimulating hormone release-inhibiting factor (MIF-1) [5], i.e. they negatively affect the inhibition of the release of melanocyte-stimulating hormone, and thus increase the concentration of melanocytes in epidermis. In relation to the effect of alaptide as a potential CPE it is important to note that melanocytes significantly influence the creation and function of keratinocytes by means of melanosomes [6–8]. During biological assays it was found that alaptide showed significant curative effect in different therapeutic areas [5], for example, it reduced the number and the extent of gastric ulcers [9,10], increased cell proliferation and epidermal regeneration and significantly accelerated regeneration (curing) of skin injuries [5,11]. Although bulk alaptide is absorbed from the gastrointestinal tract or permeates through the skin [5,12], nanonized alaptide (NALA) permeates through the skin insignificantly [12]. Alaptide is not metabolized and is excreted mostly via urine [13]. Alaptide demonstrated very low acute toxicity; no subchronic and chronic toxicity, genotoxic, teratogenic and embryotoxic effects were observed [5,14,15]. Alaptide enantiomers do not induce cytochrome P450 (1A1, 1A2, 1B1) [16].

Indomethacin, [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid, (Figure 1) is a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic and antipyretic activity. Its pharmacological effect is mediated through inhibition of cyclooxygenase (COX), the enzyme responsible for catalysing the rate-limiting step in prostaglandin synthesis via the arachidonic acid pathway. Indomethacin inhibits both isoforms COX-1 and COX-2, with greater selectivity for COX-1, which accounts for its increased adverse gastric effects relative to other NSAIDs. COX-1 is required for maintaining the protective gastric mucosal layer. The analgesic, antipyretic and anti-inflammatory effects of indomethacin occur as a result of decreased prostaglandin synthesis. Its antipyretic effects may be due to the action on the hypothalamus, resulting in an increased peripheral blood flow, vasodilation and subsequent heat dissipation. Indomethacin is commonly commercially available in oral, rectal and topical formulations [17].

This follow-up paper to the previous contributions [18–22] is aimed at the investigation of the effect of nanonized alaptide as a potential CPE on the permeation of indomethacin substance through the skin.

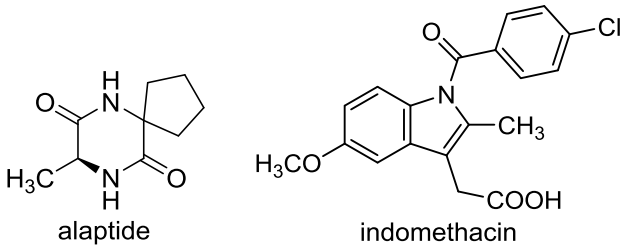

Figure 1. Structures of (S)-alaptide and indomethacin.

Experimental

Preparation of nanonized alaptide

Alaptide was synthesized by the standard process [23]. All reagents were purchased from Aldrich and Merck. The suspension of alaptide (30 g), polyvinylpyrrolidone (30 g) and purified water (240 mL, during

milling was diluted by addition of additional 150 mL) was initially mixed for 12 h at ambient temperature and then filtered through a mill sieve. The milling procedure was performed using a nanomill NETZSCH (Germany) with glass beads (0.3 mm); the rotor speed was 986 rpm; the pump speed was 30 rpm; the temperature in the grinding chamber was within 17–20 °C. The rotor speed was increased to 1500 rpm after 6 h of milling. The total time of milling was 57.5 h. The content of alaptide in the suspension was 38.76 g/L (determined by RP-HPLC [24]). The particle size x_{90} of the prepared nanonized alaptide measured by Sympatec NANOPHOX 0138 P (Germany) was 770 nm.

In vitro transdermal permeation experiments

Skin samples were obtained from porcine ear. Full-thickness pig ear skin was cut in fragments and stored at −20°C until utilized. Skin samples were slowly thawed (at 4 °C overnight and then at ambient temperature) before each experiment [25,26]. The penetration enhancing effect of NALA was evaluated *in vitro*, using a vertical Franz diffusion cell (SES – Analytical Systems, Germany) with a donor surface area of 0.6359 cm^2 and a receptor volume of 5.2 mL. The skin was mounted between the donor and receptor compartments of the Franz diffusion cell with the epidermal side up. The receptor compartment was filled with phosphate buffered saline (pH 7.4) and maintained at 34±0.5 °C [25,27], using a circulating water bath. The receptor compartment content was continuously stirred using a magnetic stirring bar. The skin was kept in contact with the receptor phase for 0.5 h prior to the experiment. Indomethacin was purchased from Sigma; all other reagents were purchased from Merck. The donor samples were prepared by dissolving indomethacin (10 mg) and NALA (1 mg of alaptide, i.e. 26 µL of suspension) in propylene glycol (0.5 mL). 0.5 mL of water was added to the mixture. This mixture was shaken vigorously and then sonicated for 10 minutes at 40 °C, then this stable system (dissolved indomethacin in enhancer emulsion) was applied to the skin surface and the donor compartment of the cell was covered by Parafilm®. The control samples were prepared in the same manner without NALA. Samples (0.5 mL) of the receptor phase were withdrawn at pre-determined time intervals (30, 60, 90, 120, 240, 360, 480, 720 and 1440 min), and the cell was refilled with an equivalent amount of fresh buffer solution. A minimum of five determinations was performed using skin fragments from a minimum of 2 animals for each composition, and the data was expressed as means ± SD. The samples were immediately analysed by HPLC.

Analysis of samples was performed using an Agilent 1200 series HPLC system, equipped with a diode array detection (DAD) system, a quaternary model pump and an automatic injector (Agilent Technologies, Germany). Data acquisition was performed using ChemStation chromatography software. A Gemini C6-Phenyl 110A 5 µm, 250×4.6 mm (Phenomenex®, USA) chromatographic column was used. The total flow of the column was 1.0 mL/min; injection was 10 µL; column temperature was 40 °C; and sample temperature was 20 °C. The detection wavelength of 260 nm was chosen, the time of analysis was 5 min. A mixture of MeOH (HPLC grade, 49.0%) with formic acid p.a. (0.2 %), acetonitrile (HPLC grade, 50.0 %) and H_2O (HPLC – Mili-Q Grade, 1.0%) was used as a mobile phase. The retention time (t_R) of indomethacin was 3.1±0.05 min; the limit of detection (LOD) was 0.059 µg/mL; and the limit of quantification (LOQ) was 0.197 µg/mL.

As a result of the sampling, the receptor compartment concentration of alaptide was corrected for sample removal and replenishment using equation: $C'_n = C_n\ (V_t/V_t\text{-}V_s)\ (C'_{n-1}/C_{n-1})$; where C'_n = corrected drug concentration in the n^{th} sample, C_n = measured drug concentration in the n^{th} sample, C'_{n-1} = corrected drug concentration in the $(n\text{-}1)^{th}$ sample, C_{n-1} = measured drug concentration in the $(n\text{-}1)^{th}$ sample, V_t = total volume of receptor solution, V_s = volume of the sample, and $C'_1 = C_1$ [28]. The corrected data were expressed as the cumulative drug permeation (Q_t) per unit of skin surface area using equation: $Q_t = C'_n/A$;

where A = 0.6359 cm^2 in our experiment. From the slope of the linear portion of the curve [29] of the dependence the cumulative amount of drug per unit area (Q_t [μg]) on time (T [h]), steady-state permeation flux (J [μg/h/cm^2]) was determined. Similarly, the lag time (T_{lag} [h]) was determined by extrapolating the linear portion of cumulative amount of permeation per unit area (Q_t) versus time (T [h]) curve to the abscissa [30]. The permeability coefficient (K_p [cm/h]) can be calculated according to $K_p = J/C_d$; where C_d = drug concentration in the donor compartment. It is assumed that under sink conditions, drug concentration in the receptor compartment is negligible compared to that in the donor compartment [31,32]. The enhancement effect was expressed as an enhancement ratio (ER) that was calculated by the formula $ER = J_{ss\text{-}x}/J_{ss\text{-}k}$; where $J_{ss\text{-}x}$ = steady-state permeation flux with CPE, $J_{ss\text{-}k}$ = steady-state permeation flux without CPE [33]. All the calculated data are listed in Tables 1 and 2 and illustrated in Figure 2.

Results and Discussion

The high toxicity and the frequent occurrence of undesirable/side effects of indomethacin, on the one hand, and significant curative action, on the other hand, brought us to an idea to formulate this substance with alaptide for its skin permeation enhancement and thus prepare less irritable therapeutic system. *In vitro* skin permeation experiments were performed using static Franz diffusion cells [27] within 24 hours. Full-thickness pig ear skin was selected for *in vitro* evaluation of permeation. This tissue is a suitable *in vitro* model of human skin [34,35], because porcine skin has shown to be histologically and biochemically similar to human skin [36]. The permeation of indomethacin through the skin without and with NALA was tested from the donor vehicle of propylene glycol/water (1:1). Previous studies have indicated that propylene glycol by itself (or a propylene glycol/water system) does not interfere with membranes [37,38].

Table 1. Cumulative permeated amounts Q_t per unit area [μg/cm^2] of indomethacin (IDM) from propylene glycol:water (1:1) without and with nanonized alaptide (NALA) as potential CPE achieved in in vitro transdermal permeation experiments using Franz diffusion cell. Q_t values are expressed as mean ± SD (n = 5 experiments).

Time [h]	Cumulative permeated amounts Q_t per unit area[μg/cm^2]	
	IDM	IDM+NALA
0.5	0.9±0.2	4.6±0.6
1.0	1.7±0.3	6.2±0.5
1.5	2.8±1.0	7.5±0.3
2.0	5.1±2.0	9.3±0.8
3.0	7.6±2.2	13.1±2.5
4.0	10.5±2.5	17.0±2.9
6.0	15.1±3.3	32.8±3.4
8.0	18.9±4.6	65.2±3.6
12.0	32.0±6.2	128.9±6.3
24.0	83.2±6.5	384.1±5.2

The values obtained from the permeation experiments were expressed as the cumulative permeated amount of the drug (Q_t [μg]) per unit of skin surface area, see Table 1. Other permeation parameters of indomethacin without and with NALA from propylene glycol:water (1:1), steady-state permeation fluxes

(J [μg/h/cm^2]), lag times (T_{lag} [h]), permeability coefficients (K_p [cm/h]) and enhancement ratio (*ER*) are mentioned in Table 2. The dependences of the cumulative permeated amount of the drug per unit of skin surface area in time are illustrated in Figure 2 that is divided into parts A and B for better lucidity.

Table 2. Permeation parameters of indomethacin (IDM) without and with nanonized alaptide (NALA) from propylene glycol:water (1:1): steady-state permeation fluxes (J), corresponding lag times (T_{lag}), permeability coefficients (K_p) and enhancement ratio (*ER*). All values are expressed as mean ± SD (n = 5 experiments).

Sample	J [μg/cm^2/h]	T_{lag} [h]	$K_p \times 10^{-3}$ [cm/h]	*ER*
IDM	2.9±0.4	1.0±0.6	0.3±0.05	–
IDM+NALA	16.1±1.2	3.9±0.2	1.6±0.1	5.6±0.4

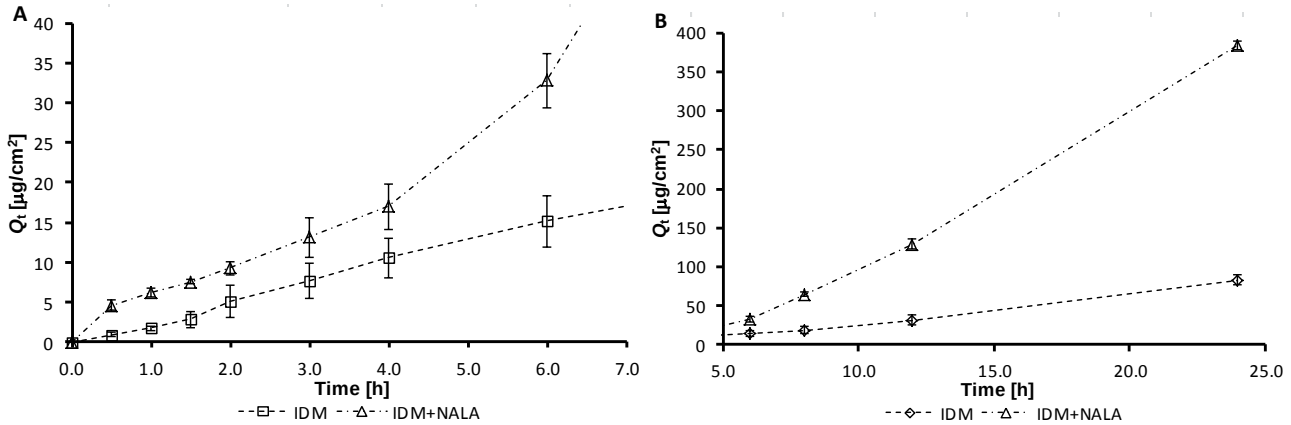

Figure 2. In vitro profile of cumulative permeated amounts Q_t per unit area [μg/cm^2] in time of indomethacin (IDM) alone and after addition of nanonized alaptide (NALA) in ratio 10:1 (IDM:NALA) from propylene glycol/water (1:1) system through skin. Q_t values are expressed as mean ± SD (n = 5 experiments).

The permeated amount of indomethacin with NALA increased rapidly already at the 30th minute, when it reached approx. 5-fold higher values than formulation without NALA, see Table 1. Similarly sharp enhancement of transdermal permeation was found, e.g., for permeation of other NSAIDs or antipyretics, such as ibuprofen, nimesulide, acetylsalicylic acid or paracetamol [18,19,21]. It can be stated that indomethacin without NALA permeated moderately in comparison with indomethacin with added NALA; in the whole investigated time range in every time the corresponding Q_t values related to the system without and with NALA were statistically different from each other, see Figures 2A and 2B. The effectivity of alaptide as a potential CPE can also be confirmed by parameters mentioned in Table 2, since significant increases of steady-state permeation flux and permeability coefficient (approx. about 5.3) were observed. Enhancement ratio calculated from steady-state permeation fluxes is 5.6, which indicates that nanonized alaptide enhanced permeation of compounds through the skin.

On the other hand, an absolutely different effect was observed for the combination of BACTROBAN® Leciva with NALA when a significant decrease of permeation (in fact, blockade of permeation) of mupirocin from ointment through the skin occurred during the experiment, indicating that mupirocin can act curatively only on the surface of the skin [20].

Based on the presented results, it can be assumed that the contribution of NALA to the enhanced permeation of indomethacin through the skin is significant not only immediately after application but also for long-term application. The structure of alaptide can be classified as a hybrid between the derivatives of

urea and 2-pyrrolidone, therefore the supposed mechanism of enhancement action can be as follows. As an urea-like derivative it can demonstrate moisturizing effect on the *stratum corneum* [38−40], and, as a 2-pyrrolidone-like derivative it can exhibit interactions preferentially in the keratin region [39,41]. Nevertheless, the exact mechanism of action of alaptide and effects of mutual interactions of indomethacin, alaptide and skin are under investigation, for example, using Raman or infrared spectroscopy for evaluation of interactions of the formulation and alaptide with the skin structure and using thermal methods for understanding of interactions between indomethacin and alaptide.

Conclusions

Modification of the permeation of indomethacin through the full-thickness pig ear skin by nanonized alaptide was investigated by *in vitro* screening using the Franz diffusion cell. Nanonized alaptide applied in ratio 1:10 related to the amount of indomethacin significantly enhanced the permeation of the drug substance from the donor vehicle of propylene glycol/water (1:1) through the skin. The calculated parameters, such as steady-state permeation flux and permeability coefficient, confirmed the enhancement effect of nanonized alaptide. Overall enhancement ratio ER is 5.6. The permeated amount of indomethacin with NALA increased rapidly already at the 30th minute, when it reached more than 5-fold higher values than indomethacin without NALA. Thus, it can be summarized that nanonized alaptide can be successfully applied as a CPE, because it provided excellent rapid onset of enhancement effect, which is an important requirement for chemical permeation enhancers. It is evident that alaptide modifies skin structure, which results in significantly enhanced permeation at long-term application.

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