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Time dependence of the enhancement effect of chemical enhancers: Molecular mechanisms of enhancing kinetics

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ABSTRACT

Chemical enhancers are widely used for facilitating drug penetration in transdermal drug delivery system (TDDS). However, there is a lack of knowledge about how the enhancement effect changes over time. In this study, on the basis of kinetic parameters of enhancement effect, molecular details of the dynamic enhancement process was described and a new hypothesis of the recovery mechanism of the skin barrier function was proposed. Using pretreated skin and flurbiprofen patch, the effects of Azone (AZ) and menthyl decanoate (MT-10) were evaluated with *in vitro* permeation experiment and further confirmed by confocal laser scanning microscopy (CLSM) and TEWL. The results showed that the enhancement ratio (*ER*) increased firstly, then reached a plateau and finally decreased. The enhancement effect of MT-10 was slower ($T_{onset,\ MT\text{-}10} > T_{onset,\ AZ}$), weaker ($ER_{max,\ MT\text{-}10} < ER_{max,\ AZ}$) and shorter ($T_{eff,\ MT\text{-}10} < T_{eff,\ AZ}$) than that of AZ. According to the results of CLSM, ATR-FTIR and molecular dynamic simulation, the dynamic enhancement effect was caused by the variation of the diffusion coefficient of intercellular lipid in the stratum corneum (SC), which was dependent on the affinity between enhancers and lipid. Moreover, the skin barrier function recovered although a large amount of enhancers still existed in the SC. Additionally, according to the results of ATR-FTIR, molecular docking and skin retention study, the dynamic effect of AZ on the skin protein only induced skin irritation but showed no influence on drug penetration, so did the amount of the enhancer. In conclusion, dynamic enhancement effect was caused by the dynamic effect of the enhancer on the SC intercellular lipid, and the skin barrier function recovered by accepting the enhancer as a new component of the lipid bilayer.

1. Introduction

As one of the successful controlled release technologies, transdermal drug delivery system (TDDS) provides numerous advantages and becomes an attractive subject in recent decades [1]. However, the skin forms a natural barrier to avoid the entrance of exogenous substances, its low permeability depends on the outermost layer, the highly-organized stratum corneum (SC), which is comprised of intercellular lipid and keratin-rich cells [2]. Many strategies have been used for facilitating drug penetration across the skin [3]. Among these methods, chemical enhancers show several benefits, such as formulation flexibility, easy processing and low cost. Several structure-activity relationship (SAR) studies revealed that the enhancing activity of enhancers mainly depends on their chemical structures [4,5]. Vávrová et al. [6] claimed the activity of amphiphilic enhancers was determined by the hydrogen-bonding sites, the size of the polar head and the length of the hydrophobic tails. Moreover, chemical enhancers were reported to decrease skin barrier resistance by affecting the lipid region of the SC or altering the secondary structures of skin proteins [6].

Ideally, an instantaneous, stable and sustained enhancement effect of chemical enhancers is desired in the drug penetration. However, Hirvonen et al. [7] demonstrated that variations on enhancing activity of Azone (AZ) and *n*-dodecanol were observed at four different treatment time. Maurya et al. [8] found that propylene glycol and oleic acid showed different enhancement effect during four-hour treatment. These results indicated that a dynamic enhancing process of chemical enhancers exists. Unfortunately, the results were only obtained through a simple pretreatment and the integrated enhancing process was still unclear. The questions of how the enhancing activity changes over time and what parameters can be used to evaluate the dynamic process are proposed. Their molecular mechanism should be illustrated on the basis of the dynamic process, which is useful to deepen our understanding about the enhancement effect of chemical enhancers.

The effectiveness and duration of transdermal preparations are directly affected by the enhancement effect of chemical enhancers which are supposed to be rapid, reproducible and predictable [9]. Generally, the onset time of an enhancer influences when the effective

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concentration of drugs can be arrived [10]. The offset time represents when the enhancer lost the activity, which influences the duration of the enhancement effect and the permeated amount of drugs. Moreover, even at the optimum concentration of an enhancer, drug efficiency is still affected by the variation of enhancement effect of the chemical enhancer. Therefore, the knowledge of the dynamic enhancement of enhancers is in great need.

Although chemical enhancers show extraordinary effect on enhancement of drug penetration, the associated safety problems are still obstacles for the application of chemical enhancers in the transdermal preparations. An ideal enhancer should be not only effective but also non-irritated. Thus, the irritation caused by enhancers should be monitored simultaneously during the dynamic process. It was generally accepted that substances initiated skin irritation by two distinct pathways. One was the direct damage to the barrier function of the SC intercellular lipid. The other one was the effect on the cells of the viable skin. The surfactant-like chemicals disturbed keratinocytes membrane, resulted in the release of cytoplasm which contained pro-inflammatory cytokine IL-1α and finally induced skin irritation. Both of the two pathways could lead to skin irritation alone or in combination [11].

Inspired by the previous researches, this paper attempted to (1) reveal the integrated time dependence of the enhancement effect of chemical enhancers, which was defined as "dynamic enhancement effect" and described by the enhancement ratio against pretreated time; (2) establish kinetic parameters which can be used to evaluate the dynamic enhancement effect of chemical enhancers; (3) explore the molecular mechanisms of the dynamic actions. In this work, the molecular mechanisms of the dynamic effect of AZ and menthyl decanoate (MT-10) were primarily explored by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, molecular docking, molecular dynamic (MD) simulation and skin retention study. This work was an important step in understanding the integrated enhancing process of chemical enhancers.

2. Materials and methods

2.1. Chemicals and animals

Flurbiprofen (FP) was supplied by Shanghai Haiqu Chemical Co., Ltd. (Shanghai, China). AZ was bought from Aladdin Reagent Co., Ltd. (Beijing, China). MT-10 was prepared with the methods previously reported by our group [12]. Isopropyl myristate (IPM) was purchased from China National Medicines Co., Ltd. (Shanghai, China). Methanol, ethanol and ethyl acetate of chromatographic grade was obtained from Concord Technology Co., Ltd. (Tianjin, China). All of other reagents were of reagent grade at least.

Male Wistar rats (180–220 g, 6–8 weeks old) were supplied by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All the procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University (no.·SYPU-IACUC-C2015-0513-202).

2.2. Preparation of FP patch

The solvent evaporation technique was used to prepare FP patch. Pressure sensitive adhesive (DURO-TAK® 87-4098, Henkel AG & Co. KGaA, Düsseldorf, Germany) and FP of 10% (w/w) were dissolved in ethanol. After mixed thoroughly with a mechanical stirrer, coating material was spread with a laboratory coating unit (SLT200, Shanghai Kaikai Co., Ltd., Shanghai, China) onto a release liner (ScotchPak™ 9744, 3M Co., St. Paul, USA), kept at the room temperature for 10 min, and then dried at 50 °C for 10 min to remove residual solvent. The patch was finally covered with backing membranes (ScotchPak™ 9700, 3 M Co., St. Paul, USA). The drug content was 1106.21 ± 16.53 μg/cm^2.

2.3. In situ skin pretreatment

The rats were anesthetized with urethane (20% w/v, 6 mL/kg, i.p.). After fastened up with abdomen upward, hair of the abdominal area was trimmed and shaved without damaging the underlying skin. After washed and dried carefully, the skin was divided into three separate sections with each area of 0.95 cm^2. One of them was used as control, the rest sections of the skin were used for the pretreatment of MT-10 or AZ. Pure AZ of 5.0 μL and pure MT-10 of 5.0 μL were spread onto the administration area separately and uniformly. The skin was disposed for 5, 15, 30, 60, 120, 180, 240, 360, 480, 720, 960, 1200, 1440 min in different groups. Four rats were used for the pretreatment at each time point ($n = 4$). Next, the residual enhancers on the surface were removed carefully and completely [13]. All of the work was carried out in a windless house with the ambient temperature of 22 ± 2 °C. After pretreated, full-thickness skins of rats were excised from the treated sites with scissors. The adhering subcutaneous tissues were removed carefully.

2.4. Dynamic skin barrier function

2.4.1. In vitro permeation experiments

Modified side-by-side diffusion cells with an effective diffusion area of 0.95 cm^2 were applied in the *in vitro* permeation study. The cells were kept at 32 °C by the circulated water bath and stirred with magnetic bars at 400 rpm. The prepared skin was mounted between two cells, with the dermal side facing toward the receptor cell. FP patch was pressed on the skin with the adhesive side facing the SC. After securely clamping the cells together, the receptor compartment was filled with phosphate buffer solution (pH 7.4) of 4.0 mL. At predetermined time points (2, 4, 6, 8, 10, 12 h), receptor solution of 2.0 mL was withdrawn and the same volume of fresh receptor medium was added into the receptor cell in order to maintain sink condition. The samples were analyzed by HPLC method described in our previous work [10].

The cumulative penetrated amount per unit area ($Q_{12\ h}$, μg·cm^{-2}) *versus* time was plotted. Enhancement ratio (*ER*) was used to evaluate the activity of the enhancers, which is the ratio of $Q_{12\ h}$ with enhancer to $Q_{12\ h}$ without enhancer.

$$ER = Q_{12\ h}(\text{with enhancer})/Q_{12\ h}\ (\text{without enhancer}) \quad (1)$$

2.4.2. Confocal laser scanning microscopy (CLSM)

CLSM was introduced to visualize the dynamic enhancement effect of chemical enhancers. The experiment procedures were carried out according to our previous study [14]. A LSM 710 Laser Scan Microscope (Carl Zeiss AG, Jena, Germany) was used for the skin imaging. An excessive fluorescein was added into IPM to prepare saturated fluorescein solution. After sonication for 10 min and centrifuged at 5000 rpm for 5 min, the supernatant was filtered with a 0.45-μm filter. The prepared skin (pretreated for 5 min, 180 min and 1440 min, respectively in Section 2.3) was mounted in the Franz diffusion cells. Fluorescein solutions of 200 μL were added into the donor cells and the receptor cells were filled with PBS of 4.0 mL. After a 20-minute exposure, the solution was removed carefully. Then the skin surface was washed gently with diluted water and dried with filter paper.

2.4.3. Transepidermal water loss (TEWL) experiments

TEWL was used to investigate the changes of skin barrier function. An open-chamber Tewameter® (TM 300, Courage & Khazaha Co., Germany) was conducted to quantify the *TEWL* value, which was calculated automatically by the software and the value was expressed in g·m^{-2}·h^{-1}. The rats ($n = 4$) were anesthetized and shaved as described in Section 2.3, and then kept in the ambient conditions (22 ± 3 °C, RH 50 ± 5%). The initial *TEWL* was determined as the primary value and was labeled as $TEWL_0$. AZ and MT-10 of 5.0 μL were spread