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Synergistic effects of ethosomes and chemical enhancers on enhancement of naloxone permeation through human skin

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The purpose of this study was to investigate the effects of ethosomes, chemical enhancers and their binary combination on the *in vitro* permeability enhancement of naloxone through human skin. Franz diffusion cells were used for the percutaneous absorption studies. Propylene glycol (PG), *N,N*-dimethyl formamide (*N,N*-DMF), *N,N*-dimethyl acetamide (*N,N*-DMA), dimethyl sulfoxide (DMSO), Azone® and polyethylene glycol 400 (PEG400), were chosen as the chemical enhancers. Naloxone ethosomes showed 11.68 times increase in steady-state flux compared to phosphate buffered solution (PBS). Ethosomes in combination with chemical enhancers synergistically increased ($p < 0.05$) *in vitro* flux of naloxone. Azone® 3% + PG7% pretreated in ethosomal form dramatically enhanced the skin permeation of naloxone *in vitro* compared with ethosomes (steady-state flux: $96.75 \pm 5.70\ \mu g \cdot cm^{-2} \cdot h^{-1}$ vs $20.56 \pm 1.67\ \mu g \cdot cm^{-2} \cdot h^{-1}$). Ethosomal carrier and enhancers accumulated in the skin after 24 h were greater than that of PBS.

There are some literature reports that penetration enhancers were effective promoters of naloxone flux (Aungst et al. 1986; Jaiswal et al. 1999; Panchagnula et al. 2001, 2005).

One of the possibilities for increasing skin permeation of drugs is the use of vesicular systems (Honeywell-Nguyen et al. 2002). Classic liposomes are of little value as carriers for transdermal drug delivery because they do not deeply penetrate the skin. Recently, ethosomes have appeared in the fields of pharmaceutical technology and drug delivery (Dayan and Touitou 2000; Godin et al. 2003; Godin et al. 2004; Touiton et al. 2000). The aim of this present study was to develop naloxone ethosomes which can enhance the skin permeation of naloxone. The synergistic effects of chemical enhancers and ethsomes were also investigated.

Propylene glycol (PG), *N,N*-dimethyl formamide (*N,N*-DMF), *N,N*-dimethyl acetamide (*N,N*-DMA), dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG400) and Azone® (3%) + PG(7%) were chosen as chemical enhancers. The epidermis of cadaver skin was pretreated with these enhancers for 40 min and then washed with deionized water. Permeation studies showed that the flux values of naloxone in the presence of these chemical enhancers were significantly increased when compared to that of control ($p < 0.05$) and varied from 2.73 to 9.57 times (Table). These results are consistent with previous reports (Jaiswal et al. 1999). The most significant enhancement is obtained with Azone® (3%) + PG(7%) followed by *N,N*-DMF and PG (Table). Enhancement ratios (ER) in the presence of the various sorption promoters were also calculated (Table).

Ethosomes, which were made up of water, phospholipids and ethanol at various concentrations were able to significantly enhance the permeation of drugs and ensure skin

Table: The steady-state flux, amount in skin and enhancement ratio of naloxone permeating through human skin

Enhancer	Steady-state flux ($\mu g \cdot cm^{-2} \cdot h^{-1}$)[c]	ER[d]	ER[e]	Amount in skin ($\mu g \cdot cm^{-2}$)[c]
PBS	1.76 ± 0.04	–	–	10.06 ± 1.23
N,N-DMF[a]	8.15 ± 0.22	4.63	–	35.32 ± 1.81
N,N-DMA[a]	4.81 ± 0.50	2.73	–	22.70 ± 2.12
DMSO[a]	6.49 ± 0.30	3.69	–	13.16 ± 1.03
PG[a]	7.78 ± 0.41	4.42	–	37.87 ± 3.67
PEG400[a]	5.17 ± 0.52	2.94	–	39.38 ± 2.75
Azone® (3%) + PG (7%)[a]	16.84 ± 0.74	9.57	–	51.08 ± 5.36
Ethosomes	20.56 ± 1.67	11.68	11.68	48.63 ± 6.02
N,N-DMF[b]	54.72 ± 2.64	31.81	6.87	82.36 ± 6.95
N,N-DMA[b]	26.35 ± 2.56	14.17	5.19	64.53 ± 3.34
DMSO[b]	43.54 ± 1.32	24.74	6.70	49.03 ± 3.87
PG[b]	46.08 ± 3.11	26.18	5.92	92.31 ± 7.29
PEG400[b]	27.63 ± 2.91	15.70	5.34	79.33 ± 5.03
Azone® (3%) + PG (7%)[b]	96.75 ± 5.70	54.97	5.74	108.28 ± 9.30

phosphate buffered solution (PBS), propylene glycol (PG), *N,N*-dimethyl formamide (*N,N*-DMF), *N,N*-dimethyl acetamide (*N,N*-DMA), dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG400)
[a] Naloxone solution with 5% enhancers (v/v)
[b] naloxone ethosomes with 5% enhancers (v/v)
[c] Mean ± S
ER[d] = the steady-state flux of each group/the flux of naloxone PBS
ER[e] = the steady-state flux of ethosomes + a given chemical enhancer/the flux of same chemical enhancer

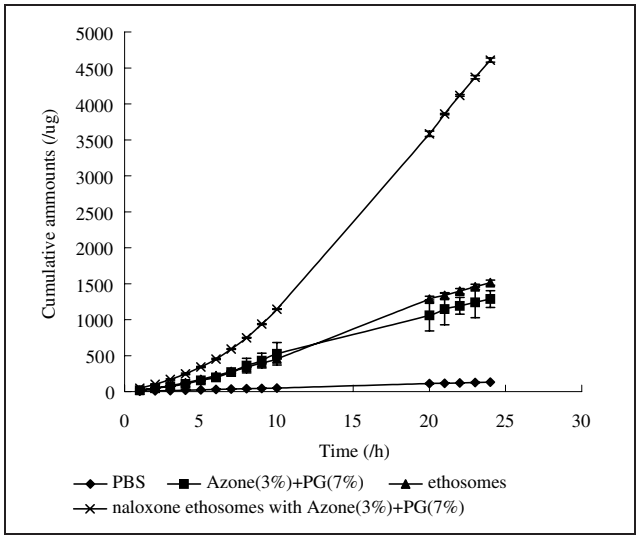

Fig.: Cummulative penetration of naloxone through human skin. Each experiment was performed in triplicate and the values were expressed as mean ± SD

accumulation (Godin and Touitou 2003; Lodzki et al. 2003; Godin and Touitou 2004). The steady-state flux of naloxone from ethosomes increased significantly in comparison to naloxone in phosphate buffered solution (PBS, Table). The results also showed that the enhancer effect of ethosomes was greater than that of chemical enhancer pretreatment. The effect of ethosomes can be explained by the fact that ethanol disturbs the organization of the stratum corneum lipid bilayer and enhances its lipid fluidity, then generates a pathway for ethosome vesicles through the skin. Fusion of ethosomes with cell membranes in deeper skin layers results in release of the drug (Touiton et al. 2000).

A simultaneous application of ethosomes and chemical enhancers significantly increased skin permeability (Table). The degrees of naloxone delivery were significantly greater than the effects of chemical enhancer alone and ethosomes alone. The flux values of naloxone ethosomes in the presence of these chemical enhancers were significantly increased when compared to that of control ($p < 0.05$) and varied from 5.19 to 6.87 times (Table).

The possible mechanism may be due to chemical enhancer molecules perturbing the lipid bilayers of the stratum corneum, thus reducing the skin barrier's fluidity, providing the vesicles with soft flexible characteristics which allow them to more easily penetrate into deeper layers of the skin, leading ethosomes to an increased rate of drug transport.

Fig. 1 clearly demonstrates the synergistic enhancement of skin permeability to naloxone when ethosomes and Azone® (3%) + PG(7%) are applied simultaneously. Application of the two enhancers resulted in enhancement ratio of ~55, whereas for ethosomes and Azone® (3%) + PG(7%) applied separately, the enhancement ratios achieved were 11.68 and 9.57, respectively.

The quantity of naloxone in the skin at the end of the 24-h experiment was also determined and is shown in the Table. It was statistically significantly greater from the ethosomal system than from PBS. The quantity for ethosomes was significantly higher, but not that much as for ethosomes with enhancers, especially ethosomes with Azone® (3%) + PG (7%). Some researchers reported that the quantity in skin from liposomes was lower than that from ethosomes (Dayan and Touitou 2000), but we found that the delivery by ethosomes was shown to be significantly enhanced relative to liposomes (date not shown). Hence, ethosomal carriers, combined with suitable enhancers may be good candidates to be developed into transdermal application used for the treatment of skin disease.

Experimental

1. Materials

Naloxone-HCl was purchased from Sigma-Aldrich company. Soybean phospholipid was bought from Lipoid company (Germany). Methanol and acetonitrile were of HPLC grade and all purchased from Fisher Scientific (Pittsburgh, PA, USA). All other reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

2. Procedures

Human cadaver skin from the chest, back, and abdominal regions was obtained from Zhejiang University anatomical teaching – research section and stored in a refrigerator at −77 °C. The epidermis was separated from the full-thickness tissue after immersion in 37 °C water for 30 min.

Ethosomes were prepared as described as reported elsewhere with modification (Jaiswal et al. 1999). Briefly, 300 mg soybean phospholipid and drug were dissolved in ethanol to receive ethosomal systems containing 3% phospholipids, 30% ethanol and 0.5% naloxone. Distilled water was added slowly in a fine stream with constant magnetic stirring the liquid was in a well-sealed container. Mixing was continued for an additional 5 min at room temperature, and the liquid was then filtered through a 0.45 μm microporous membrane.

3. HPLC assay for naloxone

The HPLC system (Shimadzu™, Japan) consisted of a pump (LC-10ATvp pump), and a wavelength UV detector (SPD-10Avp). Chromatography for separation and determination were carried out by applying the samples to a prepacked 5 μm (4.6 mm × 250 mm, i.d.) Diamonsil C_{18} column (Dikma, Beijing, China) at 35 °C. The analytical column was protected by a Phenomenex C_{18} guard column (4 mm × 3.0 mm, i.d.). For instrument control, data acquisition and processing, a N2000 software chromatography workstation (purchased from ZheJiang University, China) was used.

Mobile phase comprising of 0.1 M phosphate buffer solution (PH = 4.0), methanol and acetonitrile in the proportion of 75 : 15 : 10, v/v/v was pumped at a flow rate of 1 ml/min. The mobile phase was filtered through 0.45 μm nylon filters (Millipore, USA) and degassed. Samples were injected at 20 μl of injection volume and were analyzed at a wavelength of 240 nm.

4. Skin penetration

Permeation studies were carried out in one-chamber modified Franz diffusion cells (Shanghai xiekai trade limited company) composed of receiver compartments of 7 ml with effective diffusion area of 2.8 cm^2. The enhancer pretreated or untreated skin was sandwiched between the receptor and donor cells with the SC-side facing donor cell. The skin was equilibrated for 1 h with the receptor medium (pH 7.4 phosphate buffer solution). Naloxone solution or ethosomes were applied to the SC side in each donor cell, and covered with paraffin film. The cells were maintained at 37 ± 0.2 °C in water. The contents of the receiver cell were stirred with a magnetic stir bar at 200 rpm. At appropriate times, samples were entirely withdrawn from the receiver cell. An equivalent amount of normal pH 7.4 phosphate buffer solution (7 mL) was added to the receiver compartment to maintain a constant volume. A minimum of three permeation experiments were performed for each enhancer/drug combination. The samples from the receptor medium were analyzed by HPLC. The results were expressed as the mean ± SD.

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Influence of metal cations on the solubility of fluoroquinolones

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Although a clinically relevant interaction between a fluoroquinolone and a metal cation was first described more than 20 years ago the biopharmaceutical mechanism of this interaction is still not understood. One of the obvious disagreements in the literature is about the effect of metal cations on the solubility of fluoroquinolones. Namely, metal cations are reported to increase the solubility of fluoroquinolones as well as to decrease it and thus cause the lowered bioavailability. Thus in this work the solubility of ciprofloxacin, norfloxacin and ofloxacin and the effect of metal cations on the solubility of these fluoroquinolones in aqueous media of different pH values were reevaluated. The results clearly show that the metal cations either do not affect or even increase the solubility of fluoroquinolones. Thus they surely do not influence the bioavailability of these drugs by decreasing their solubility. Additionally, possible explanations for the contradictory results reported in the literature are given.

A case of a clinically significant interaction between a fluoroquinolone antimicrobial agent and metal cations present in antacids, mineral supplements and food was first reported in 1985. G. Höffken et al. have observed a decrease of ciprofloxacin bioavailability caused by a coadministration of a magnesium and aluminium containing antacid. The authors of this first report have speculated that the described drug-drug interaction may be related to the formation of complexes (coordination compounds) between the fluoroquinolone and the metal cation (Höffken et al. 1985). Numerous clinical studies in which the authors investigated drug-drug interactions between fluoroquinolones and preparations containing metal cations followed. It was shown repeatedly that the coadministration of fluoroquinolones and preparations containing metal cations generally results in a gross decrease of the fluoroquinolone bioavailability (Lomaestro and Bailie 1995).

Fluoroquinolone coordination chemistry was also intensively studied (Turel 2002). However, these studies could not confirm that the loss of fluoroquinolone bioavailability is a direct consequence of complexation with metal cations. Physico-chemical properties of fluoroquinolones in the presence of metal cations were studied to a lesser