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Research paper

Effects of penetration enhancers on Shuangwu traumatic formula: *In vitro* percutaneous absorption and *in vivo* pharmacodynamic evaluation of an herb medicine

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ABSTRACT

The purposes of this study were to improve the transdermal permeation of the Shangwu traumatic formula by chemical penetration enhancers and to investigate the pharmacodynamic changes of the formula caused by incorporated enhancers. The effects of different enhancers on the transdermal absorption of piperine, the representative component of formula, were investigated by *in vitro* permeation studies. The tests showed an increasing enhancement effect in the following order: Azone/N-methylpyrrolidone (NMP) > oleic acid > Azone/peppermint oil > Azone/oleic acid > Azone/propylene glycol > Azone > peppermint oil > NMP > propylene glycol. The ratio and the content of the most effective enhancer Azone/NMP were determined subsequently. The results suggested that the most significant penetration enhancement was achieved by 3% (w/w) Azone/NMP (3:7). Furthermore, the *in vivo* pharmacodynamic responses of the formula suspension with or without Azone/NMP were compared using hot-plate assay and xylene-induced ears edema test as models. The data indicated that the formula had positive effect on analgesis and anti-inflammatory, which can be enhanced with the addition of enhancers.

1. Introduction

Over the past two decades, soft tissue injuries (STI) have hit the spotlight. To combat the debilitating of STI, many new technologies [1–3], such as continuous passive motion, cryotherapy and ultrasound therapy, have been developed. But some traditional treatments widely used in folks should not be ignored.

Herbal medicines have been used to treat STI for thousands of years in China, and its practical and effective effect have also been demonstrated already. One of such herbal medicines is Shangwu traumatic formula (STF). The formula is composed of 20 herbs, including *Radix aconiti*, *Radix aconiti Kusnezoffii*, *Rhizoma pinelliae*, *Rhizoma arisaematis*, *Fructus piperis*, *Fructus piperis Longi*, *Lignum dalbergiae Odoriferae*, *Radix notoginseng*, *Cortex cinnamomi*, *Radix angelicae Dahuricae*, *Asarumsieboldii Mi*$_q$., *Radix et Rhizoma Nardostachyos*, *Myrrha*, *Olibanum*, *Rhizoma zingiberis*, *Acacia catechu*, *Camphora*, *Borneolum syntheticum*, *Rhizoma kaempferiae*, *Eugenia caryophyllata Thunb.* Each herb has specific property, and the combination can produce a synergistic effect of activating blood circulation, reducing swelling and alleviating pain. When applied externally, the formula can reach good curative effect.

However, the external application was restricted by its low permeability through the skin. The major obstacle to drug administration through the skin is the presence of the stratum corneum (SC), the outermost layer of the skin. Many strategies have been suggested to overcome this barrier. A popular approach is the use of chemical penetration enhancers which can increase drug absorption either into the systemic circulation or to the deeper, viable skin layers [4].

The aim of this work was to identify a suitable penetration enhancer for the STF. Thus, enhancing effects of Azone, N-methylpyrrolidone, propylene glycol, oleic acid, peppermint oil and their binary enhancer combination on the permeation of the STF were evaluated with percutaneous experiment *in vitro*. But as an herbal medicine composed of 20 herbs, quantitative analysis seems to be a difficult task due to the complexity of its constituents; therefore, one or two major components were chosen as a deputy to evaluate the penetration enhancement. Piperine, otherwise known as $C_{17}H_{19}NO_3$, is an active component of black pepper (*Fructus piperis*) and long pepper (*Fructus piperis Longi*). This chemical compound is high-content

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and easy to be detected by high-performance liquid chromatography (HPLC), and it is, therefore, chosen as the representative component of the STF to quantify.

A question was then aroused: "Are the results obtained just relying on piperine correct?" To answer this question, the anti-inflammatory and analgesic effect of the STF was evaluated to document whether the medicine, when incorporated the optimal enhancers selected according to piperine, can exhibit a greater inhibition on edema and pain.

2. Materials and methods

2.1. Chemicals and reagents

Oleic acid, Azone, propylene glycol and peppermint oil were purchased from *Sinopharm* Chemical Reagent Co. Ltd. of China. N-methylpyrrolidone was provided by ISP. All chemicals and solvents were of analytical grade.

2.2. Preparation of STF suspension

The formula was applied as a suspension. All crude drugs were obtained from *Cixi Traumatocomium*. Each crude drug was powdered with an average particle diameter of 125 ± 5.8 μm and mixed according to the formula dosage. Then, all herbal powder was well dispersed into double-weight water for future use.

2.3. Animals

Kunming mice, weighing 18–20 g (Shanghai Exp. Animals, Shanghai, China), were purchased and housed in a group of 10 animals. They were kept in a room maintained at 18–22 °C with free access to a standard laboratory diet and tap water. All experiments were conducted in accordance with Guide to the Care and Use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki. The allocation of animals to various groups was performed under randomization.

2.4. Skin membrane preparation

The abdominal hair of male mice was removed using depilatory paste 24 h before treatment. After anaesthetizing the rat with ether, the abdominal skin was surgically removed from the animal, and adhering subcutaneous fat was carefully cleaned with normal saline [6]. Then, the skin was frozen to −20 °C and covered with aluminum foil for future use.

2.5. Skin permeation study in vitro

The permeation studies were performed in Franz-type diffusion cells with a diffusion area of 3.14 cm^2. The skin was situated between the donor and receptor chambers of the cell with the dermal side in contact with reception medium. The STF suspension (4 g) was placed in the donor compartment and each receptor chamber was filled with 6 ml of receptor medium kept at 32 ± 0.5 °C by a circulating water jacket. The previous test showed that 30% ethanol in normal saline was an ideal receptor phase for piperine to attain the sink condition. All samples were withdrawn from the receptor compartment at 0.5, 1, 2, 4, 6, 8, 10 and 12 h and replaced with the same volume of the reception medium at 32 °C [7]. Five parallel determinations were performed.

The piperine content of the samples in the receptor compartment was analyzed by HPLC (Shimadzu Corp.). The assay system comprised a liquid chromatograph (LC-10Atvp) and a UV–vis detector (SPD-10A). The analyses were performed at room temperature with a Diamonsil C18 column (5 μm particle diameter, 4.6 mm × 250 mm). The mobile phase, consisting of methanol/water (80:20), was pumped at 1 ml/min. The wavelength was 343 nm, and the volume of injection was 20 μl. Calibrations were made by the external standard method.

The accumulated amount penetrated through the unit diffusion surface (Q) was calculated and plotted versus time. The steady-state flux (J) was estimated from the slope rate of the penetration curves. The ER was the ratio of J with and without the enhancer.

2.6. Penetration enhancer optimization

Azone, N-methylpyrrolidone (NMP), propylene glycol (PG), oleic acid (OA), Peppermint oil (PO) and the binary enhancer combination (1:1) including Azone/NMP, Azone/PG, Azone/OA, Azone/PO were used to improve the skin permeation of the STF suspension. To eliminate the effect of lag time, skin sample was defrosted in 37 °C saline solution 12 h before permeation experiment. Then, the stratum corneum side was covered with sterile gauze wet with 0.5 ml of different enhancers and kept in a fresh cabinet until the stratum corneum contacted with enhancers all around for 12 h [8]. Remove the gauze and wash the skin with normal saline for the permeation experiment immediately.

For a better evaluation of the benefit of the optimal penetration enhancer on the piperine of the STF, different enhancer contents (1%, 3% and 5%, w/w) were investigated. And, if the optimum enhancer was a binary enhancer combination, the ratio of enhancer to co-enhancer (3:7, 5:5, 7:3) was observed correspondingly when the whole content was fixed at 3%. The only difference, in comparison with the permeation study above, was that the enhancers of different ratios and content were added into the STF suspension directly without pretreatment.

2.7. Hot-plate assay

The 'hot-plate' analgesic method employed in this study was modified from those described in detail by Eddy and Leimback [9]. Each female mouse weighing 18 ∼ 22 g was placed on a 55 ± 1 °C hot-plate to observe its pain responses (hind-paw-licking or jumping). Each mouse was first habituated to the hot-plate twice, and the latent time before the occurrence of the pain response was recorded. Untreated mice with a background latent response time shorter than 5 s or longer than 30 s were excluded from the study. The dorsal hairs of each mouse were removed using depilatory paste 24 h before treatment. Mice were divided into two groups of 10 mice each. Group A was given just the STF suspension, while Group B was given the STF suspension mixed with optimal enhancer selected earlier. The suspension (0.8 g each mouse) was coated on the naked skin with an area of 2.5 × 4 cm^2, and the pain response time of two groups was determined 1, 2, 4, 6 and 8 h after transdermal administration. A cutoff time of 60 s was selected to avoid tissue damage. The percentage of pain response time elevation was calculated as Eq. (1).

$$\text{Elevation } (\%) = (\text{time}_{\text{after}}/\text{time}_{\text{before}} - 1) \times 100 \quad (1)$$

The $\text{time}_{\text{after}}$ is the mean pain response time observed at predetermined time after drug application, and the $\text{time}_{\text{before}}$ is the mean pain response time observed before medicine administration.

2.8. Xylene-induced ear edema in mice

The test procedure was carried out as described by Hosseinazadeh et al. [10]. In each mouse weighing 20 ± 2 g, the drug sample (50 mg each mouse) was besmeared on the right ear 0.5 h after the induction of ear edema by topical application of 0.02 ml xylene on both surfaces of the right ear. The left ear without any treatment

served as a control. Mice were divided into three groups of forty mice each. The experimental Group A and B were given the STF suspension with and without optimal enhancer selected earlier, respectively, while the control group was treated with normal saline. Eight mice of each group were sacrificed by cervical dislocation 1, 2, 4, 6 and 8 h after administration. Ear disks of 8.0 mm diameter were punched out and weighed. The extent of edema was evaluated by the weight difference between the right and the left ear disks of the same animal. The percentage of edema inhibition was calculated as Eq. (2).

$$\text{Inhibition } (\%) = (1 - \text{swelling}_{\text{treated}}/\text{swelling}_{\text{control}}) \times 100 \qquad (2)$$

The $\text{swelling}_{\text{treated}}$ is the mean extent of edema observed in two experimental groups, and the $\text{swelling}_{\text{control}}$ is the mean extent of edema observed in the control group.

2.9. Statistical analysis

The data were presented as mean ± SD. Statistical significance between control and treated groups was tested by ANONA.

3. Results

3.1. Penetration enhancer optimization

The cumulative amount of piperine, shown in Fig. 1, increased linearly progressively with time. With the addition of different enhancers, the permeation of piperine was remarkably enhanced compared with that of the control group ($P < 0.05$), except propylene glycol ($P > 0.05$). Table 1 showed the rank order of enhancement effect on piperine in the STF suspension was Azone/NMP > OA > Azone/PO > Azone/OA > Azone/PG > Azone > PO > NMP. The most outstanding penetration enhancer was Azone/NMP, which had the steady-state flux up to 4.54 ± 0.57 μg cm^{-2} h^{-1}, providing an almost 4.17-fold increase in permeation coefficient, followed by OA with a 3-fold increase.

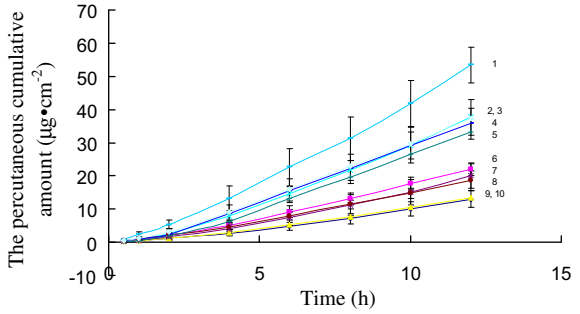

Fig. 1. The percutaneous cumulative amount of piperine across rat skin pretreated with different penetration enhancers ($n = 5$). (1) Azone + NMP, (2) OA, (3) Azone + PO, (4) Azone + OA, (5) Azone + PG, (6) Azone, (7) PO, (8) NMP, (9) PG and (10) blank.

Table 2
The permeation kinetic parameters of piperine with different ratio of NMP/Azone when the content is fixed to 3% (mean ± SD; $n = 5$).

NMP:Azone	Permeation equation	r	J (μg cm^{-2} h^{-1})	ER
Control	$y = 1.0851x - 0.9447$	0.9903	1.09 ± 0.22	
3:7	$y = 1.7761x - 1.3028$	0.9986	1.78 ± 0.21$^{*,▲▲}$	1.64
5:5	$y = 1.923x - 0.9807$	0.9970	1.94 ± 0.33**	1.77
7:3	$i = 2.2641x - 1.2676$	0.9986	2.18 ± 0.29$^{**,▲}$	2.09

Compared with control.
Compared with group 5:5.
* $P < 0.05$.
** $P < 0.01$.
$^{▲}$ $P < 0.1$.
$^{▲▲}$ $P < 0.05$.

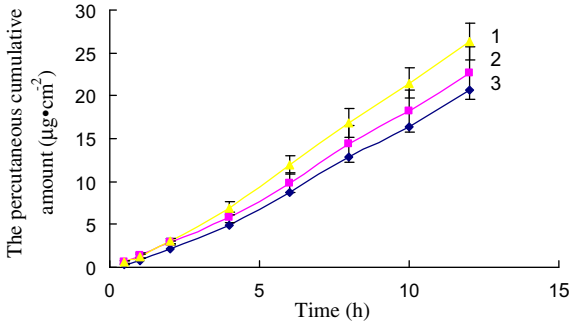

Fig. 2. The permeation curves of piperine with different ratio of NMP and Azone when the content is fixed to 3% ($n = 5$). (1) Azone:NMP (3:7); (2) Azone:NMP (5:5) and (3) Azone:NMP (7:3).

Having confirmed Azone/NMP as the optimum penetration enhancer, the optimum rate of Azone and NMP was further investigated. The results indicated that the flux of piperine increased with the increasing proportion of NMP in the binary enhancer combination as shown in Table 2 and Fig. 2. Statistical difference ($P < 0.1$) was found between 5:5 (Azone to NMP) group and 3:7 group and, therefore, the highest permeation rate was achieved with Azone to NMP as 3:7.

The effects of Azone/NMP concentration were demonstrated in Fig. 3, and skin permeation parameters were listed in Table 3. The cumulative amount and permeation coefficient increased with the concentration increasing from 1% to 5%, but no statistically significant difference was found between 3% group and 5% group

Table 1
The permeation parameters of piperine with different penetration enhancers (mean ± SD; $n = 5$).

Groups	Permeation equation	r	J (μg cm^{-2} h^{-1})	ER
Control	$y = 1.0851x - 0.9447$	0.9903	1.09 ± 0.22	
Azone	$y = 1.8917x - 1.5118$	0.9968	1.90 ± 0.17**	1.74
Propylene glycol	$y = 1.1388x - 1.0026$	0.9941	1.15 ± 0.18	1.05
Oleic acid	$y = 3.2536x - 3.3563$	0.9939	3.26 ± 0.47**	3.00
Peppermint oil	$y = 1.6897x - 1.6783$	0.9918	1.70 ± 0.34**	1.56
N-methylpyrrolidone	$y = 1.5706x - 0.9265$	0.9971	1.58 ± 0.23*	1.45
Azone/PG	$y = 2.9375x - 3.2819$	0.9935	2.95 ± 0.60**	2.71
Azone/OA	$y = 3.1563x - 2.7746$	0.9972	3.17 ± 0.77**	2.91
Azone/NMP	$y = 4.5262x - 3.2399$	0.9967	4.54 ± 0.57**	4.17
Azone/PO	$y = 3.2285x - 2.5447$	0.9973	3.24 ± 0.46**	2.98

Compared with control group.
* $P < 0.05$.
** $P < 0.01$.

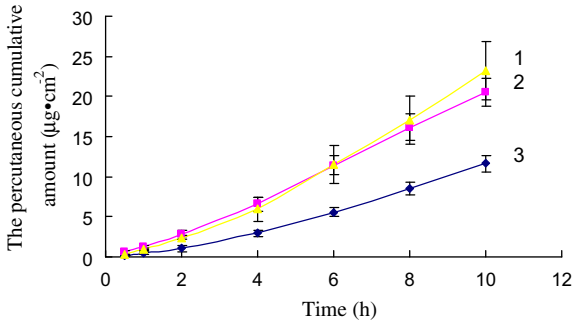

Fig. 3. The permeation curves of piperine with different contents of NMP/Azone when the ratio is fixed to 7:3 (n = 5). (1) 5% enhancers; (2) 3% enhancers and (3) 1% enhancers.

Table 3
The permeation kinetic parameters of piperine with different contents of NMP/Azone when the ratio is fixed to 7:3 (mean ± SD; n = 5).

NMP/Azone concentration	Permeation equation	r	J (μg cm^{-2} h^{-1})	ER
Control	$y = 1.0851x - 0.9447$	0.9903	1.09 ± 0.22	
1%	$y = 1.2701x - 1.2921$	0.9932	1.28 ± 0.10▲	1.17
3%	$y = 2.1709x - 1.232$	0.9986	2.18 ± 0.29**	2.00
5%	$y = 2.5157x - 2.3927$	0.9944	2.52 ± 0.36**	2.32

Compared with control.
Compared with group 3%.
** $P < 0.01$.
▲ $P < 0.05$.

($P > 0.05$). Thus, 3% was a suitable concentration. According to the results above, the most outstanding penetration enhancer was Azone/NMP (3:7) at 3% (w/w).

3.2. Hot-plate assay

As shown in Table 4, the STF suspension with and without Azone/NMP enhancer both exhibited a significant ($P < 0.05$) increase in the latency to response to hot-plate thermal stimulation, but the addition of enhancer produced a more significant ($P < 0.05$) elevation at different time after administration. The analgesic effect increased with the prolongation of administration (Fig. 4).

3.3. Xylene-induced ear edema in mice

Compared with the control group, the STF suspension with and without Azone/NMP enhancer showed a significant inhibition effect to the ear edema induced by xylene ($P < 0.05$), respectively (Table 5). The inhibition was increased with the addition of

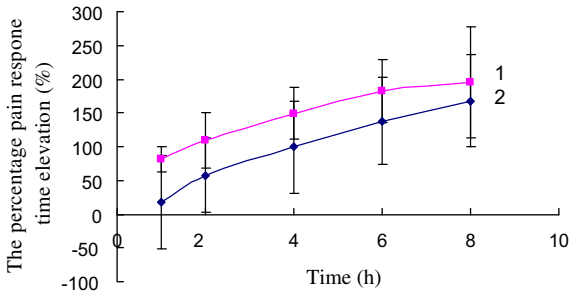

Fig. 4. The percentage pain response time elevation after drug application (n = 10). (1) Drug + enhancers (Group B) and (2) drug (Group A).

enhancers ($P < 0.1$), which showed obviously from the distance of two lines in Fig. 5.

4. Discussion and conclusion

4.1. In vitro animal model for percutaneous absorption

Since enough human skin for large numbers of permeability experiments is usually not available, various studies have been carried out in an attempt to correlate *in vitro* permeation data in animal and human skin. Rat skin has a SC that is in fact as thick as that of human skin, so the skin of rat is a feasible model for permeability experiments. A focus of several reports was to compare transdermal permeation kinetics between rodent and human skin [11]. The data suggested that rat skin was more permeable to all tested substances than human skin. Ravenzwaay et al. [12] evaluated transport of compounds with various lipophilicities across rat and human skins *in vitro*. In all cases, the penetration permeation flux through rat skin was approximately 11-fold more permeable than human skin. Based on this proportion, the approximate percutaneous permeation flux through human skin can be predicted from *in vitro* permeation studies through excised rat skin.

4.2. Penetration enhancer

Azone is an effective permeation enhancer for both hydrophilic and hydrophobic drug and did enhance skin penetration and retention of a steroid to a significant degree ($P < 0.05$), Beastall et al. reported that Azone can increase the fluidity of the intercellular lipid bilayers of the SC and lower of phase transition temperature, thereby encouraging disruption of lipid bilayers to enhance drug skin penetration [13]. The efficacy of triamcinolone preparations with and without Azone was assessed in clinical studies of atopic dermatitis. Patient treated with triamcinolone plus Azone showed greater improvement at day 15 than those

Table 4
The pain response time after drug application (mean ± SD; n = 10).

Group	The pain response time (s)					
	0 h	1 h	2 h	4 h	6 h	8 h
A	19.83 ± 4.44	23.47 ± 6.35*	31.00 ± 8.93**	39.10 ± 9.78**	46.28 ± 9.41**	50.53 ± 8.88**
B	19.90 ± 3.78	34.47 ± 8.72**,▲▲	40.51 ± 8.45**,▲	47.69 ± 7.83**,▲	54.00 ± 6.95**,▲	56.20 ± 3.78**,▲

Compared with 0 h.
Compared with Group A.
* $P < 0.05$.
** $P < 0.01$.
▲ $P < 0.05$.
▲▲ $P < 0.01$.

Table 5
The extent of edema after drug application (mean ± SD; $n = 8$).

Group	The extent of edema (mg)				
	1 h	2 h	4 h	6 h	8 h
Control	23.83 ± 2.59	19.41 ± 1.65	16.15 ± 3.70	9.28 ± 3.98	7.90 ± 3.27
A	15.69 ± 1.80**	12.65 ± 2.10**	10.51 ± 5.03*	5.76 ± 3.62*	4.51 ± 2.24**
B	12.86 ± 2.20**,▲▲	10.04 ± 3.56**,▲▲	7.28 ± 3.53**,▲	3.54 ± 2.82*,▲▲	2.76 ± 2.29**,▲

Compared with control.
Compared with Group A.
* $P < 0.05$.
** $P < 0.01$.
▲ $P < 0.1$.
▲▲ $P < 0.05$.

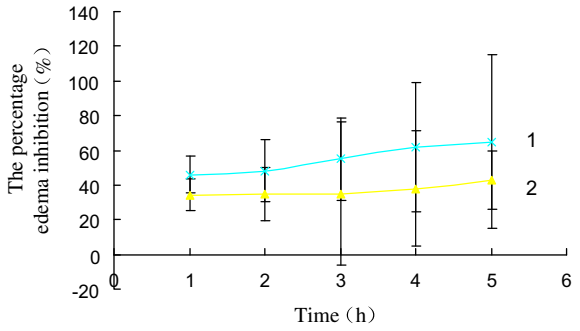

Fig. 5. The percentage edema inhibition after drug application ($n = 8$). (1) Drug + enhancers (Group B) and (2) drug (Group A).

treated with triamcinolone alone, and the difference was statistically significant [14].

Enhancing effects of Azone alone or in combination with NMP, PO, PG and OA on the permeation of the STF were evaluated in this article. The results showed that Azone/NMP provided a most excellent penetration enhancement to piperine, and the ability increased with the increasing proportation of NMP in binary enhancer. The precise mechanism has not been unclear. It was more likely that NMP, as a efficient solubilizer, could improve the penetration of Azone into the SC and then Azone in the SC took effect to enhance drug skin penetration. Further investigation is required to elucidate the exact mechanism, and clinical application of Azone/NMP is needed to confirm the clinical therapeutic effects.

The oleic acid displayed a second positive effect on piperine permeation through a dual mechanism as suggested by Aarti et al. [15] involving lipid perturbation via both conformational permutations and phase separation, with the latter effect predominating. However, when OA was used in combination with Azone, no synergistic enhancement on the piperine permeation was observed, which is contrary to the other binary enhancer combination.

4.3. Pharmacodynamic study

As two main effects of the STF are swelling reduction and pain release, the anti-inflammatory and analgesic effects of the STF suspension were investigated in this study. The hot-plate thermal stimulation was selected to study the central analgesic action mediated through inhibition of central pain receptors possibly [16]. Xylene-induced ear edema in mice was selected to reflect the edematization during the early stages of acute inflammation. The mediators can induce ear edema by promoting vasodilation and increasing vascular permeability [17]. Experimental evidence obtained in the present study indicated that external use of the STF suspension had a significant anti-inflammatory and analgesic effect on these two models, which enriched our understanding of folk use of the STF in treating STI.

4.4. Enhanced pharmacodynamic effect with the addition of optimal enhancer

In this study, the pharmacodynamic changes caused by incorporated optimal penetration enhancer were compared to validate the authenticity of *in vitro* percutaneous results. The incorporation of the optimal enhancer produced an improvement of both anti-inflammatory and analgesic effect. Enhancing effects showed obviously from the distance of activity–response lines in Figs. 4 and 5. The greater pharmacodynamic activities, to some extent, were due to a penetration enhancement of the STF, which meant that the optimally selected enhancer just relying on the representative component can improve the transdermal permeation of the whole formula. This conclusion demonstrated that the data optimized on the basis of quality control of some active components through in vitro penetration experiment can predict *in vivo* skin penetration status of the overall formula. Based on this research, we tried to provide a possible method to evaluate the skin permeation evaluation of complex prescriptions by combining in vitro percutaneous experiment with *in vivo* pharmacodynamic evaluation.

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