in vitro Studies on Transdermal Absorption of Extract from *Daphnes Giraldii* Cortex

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Received: 9 August 2013;

Accepted: 28 October 2013;

Published online: 16 July 2014;

AJC-15553

The percutaneous permeability of three major components (daphnin, daphnetin-8-O- β -D-glucoside, daphnetin) in the extract from *daphnes giraldii* cortex (the dried stem bark and root bark of *daphne giraldii*, *daphne retusa* and *daphne tangutica*) simultaneously are investigated and established the foundation for the development of transdermal delivery system of babu plaster of *daphnes giraldii* cortex extract. The franz diffusion cell method and razor processed excised rabbit skin were used to evaluate the transdermal absorption characteristics of *daphnes giraldii* cortex extract. The effects of enhancer dosage and drug loading on the percutaneous permeability were studied. The penetration rate constant (Js) was 14.9276 $\mu\text{g}/(\text{cm}^2 \text{ h})$ under the optimum condition. Fitting results showed that the penetration curve was related to zero level kinetic equation. This study has built the *in vitro* percutaneous analysis method for the active components in the extract from *daphnes giraldii* cortex, *daphnes giraldii* cortex extract has the good percutaneous permeability which suggests that it is suitable for the transdermal delivery system in the treatment of rheumatic diseases.

Keywords: Daphnin, Daphnetin-8-O- β -D-glucoside, Daphnetin, *in vitro*, HPLC.

INTRODUCTION

Rheumatosis is recognized as a particular disease with an extremely high incidence in medical field. Research showed that 80 % of the patients with rheumatic diseases run the risk of cardiovascular, lung and kidney diseases, which directly endanger their life. In our country, there are up to 230 million people who suffer from various rheumatic diseases, nearly 80 million of them are living by a crutching or wheelchair-bound life. Therefore, it is necessary to develop the effective therapeutic method for rheumatic diseases.

Celecoxib capsules as well as methylprednisolone tablets and other western medicine are the major drugs for the treatment of rheumatic diseases in the market. Chinese materia medica has a permanent cure compared with the western medicine, therefore there is an increasing potential of Chinese materia medica used with the outstanding efficacies.

Daphnes chinese materia medica *giraldii* cortex, clinically used for the treatment of rheumatism, arthritis and other similar diseases¹, is the dried stem and root bark of *daphne giraldii* nitsche, *daphne retusa* hemsl. and *daphne tangutica* maxim (thymelaeaceae). The main chemical components in the extract of *daphnes giraldii* cortex are coumarin compounds²⁻⁵,

diterpenoids⁶⁻⁸, lignans, flavonoids, anthraquinone and sterols, etc. In order to increase the concentration of the active components, enhance the antiinflammatory effects and reduce the systemic adverse reactions, we extracted three active components (daphnin, daphnetin-8-O- β -D-glucoside, daphnetin) from the extract of *daphnes giraldii* cortex.

There are many drug delivery systems, but the transdermal drug delivery system can overcome the peak valley fluctuation phenomenon and reduce the side effects, so it is the ideal way. Therefore we made the transdermal delivery system of babu plaster of *daphnes giraldii* cortex extract. Because the major barrier of drug percutaneous absorption is stratum corneum, we investigated the transdermal characteristics of the active components from the extract of *daphnes giraldii* cortex *in vitro* and developed the simultaneous determination method for them. We also studied the effects of drug loading and enhancer dosage on the transdermal characteristics.

There is small amount of poison in the extract of *daphnes giraldii* cortex, which leads to its slightly poisonous effect^{9,10}. The accumulative permeation ratio can be largely increased both for the active and the poisonous components with the action of penetration enhancer¹¹. So we investigated the penetration enhancement function of azone with the major

purpose of estimating the optimum dosage and providing the basis for the acute dosage of the slightly toxic extract from daphnes giraldii cortex. In this study, we investigated the permeation characteristics of the three active components which are different from each other on the account of different permeation enhancement function caused by difference in polarity¹⁰. Therefore in our research, we confirmed that the permeation enhancement function of azone was different for the different polarities. As demonstrated in reference documentation¹², the *in vitro* permeation data and the *in vivo* pharmacokinetics can be correlated by deconvolution, thus our study can also provide a basis for the *in vivo* pharmacokinetics of daphnes giraldii cortex extract.

To date, there has been no research paper on the simultaneous detection of the three active components, daphnin, daphnetin-8-O- β -D-glucoside, daphnetin, from the extract of daphnes giraldii cortex. In our research, we developed a simultaneous detection for the three active components for the first time.

EXPERIMENTAL

Transdermal Diffusion Instrument (TK-12B, Shanghai Kai Kai Technology Trading Co., Ltd, Shanghai, China), Agilent 1100 high performance liquid chromatography, Analytical Balance (AB204-N Mettler Toledo Science and Technology Co., Ltd, Shanghai, China).

Daphnetin National Institutes for Food and Drug Control, batch number: 0900-20001, content > 98 %; daphnin (homemade, content > 98 %), daphnetin-8-O- β -D-glycoside (homemade, content > 98 %); methanol (of chromatographic purity, Tianjin Concord Science and Technology Co., Ltd., Tianjin, China); phosphoric acid (of analysis purity, Tianjin Concord science and Technology Co., Ltd, Tianjin, China); daphnes giraldii cortex extract (homemade, batch number: 20101124); Babu plaster of daphnes giraldii cortex extract (homemade); Razor (Philishave QG3040); Sodium sulfide (of analysis purity, Tianjin Fengchuan Chemical Technology Co., Ltd, Tianjin, China. Batch number: 200911300); Azone (Ruicheng Dongtai Intermediates Co., Ltd, Shanxi, China).

Animal: Rabbit ♀, (2.0 $\pm$ 0.5) kg was procured from the Tianjin Institute of Drug Safety Evaluation Center, Tianjin, China.

Processing method of excised skin: The back hair of anesthetized rabbit was shaved and rinsed. Then, after 24 h, the back skin of executed rabbits was cut and fat and tissue were peeled away.

***in vitro* Transdermal absorption:** The excised skin was cut into 3 pieces with appropriate size, then placed between the three supply tanks and the receiving pools of the transdermal diffusion instrument. Babu plaster of daphnes giraldii cortex extract (2.54 cm²) was stuck on the epidermal layer of the skin and was fastened with iron clamp. The receiving pools (6 mL in total) were filled with ethanol-saline (1:9) solution. The epidermal layer was put towards the supply chamber and the dermis layer was put towards the receiving chamber.

The thermostatic water bath circulation (37 $\pm$ 1) °C and magnetic heating stirrer at 200 rpm were actuated. The sample (1 mL) was taken at 2, 4, 6, 8, 10 and 24 h, respectively, then

added with the same volume of fresh ethanol-saline (1:9) solution at 37 °C, respectively¹³.

The concentration of receiving liquid was determined and the accumulative permeation amount (Q_n , $\mu\text{g}/\text{cm}^2$) was calculated according to the eqn (1). The Q_n and time (t_h) were calculated by linear regression. The slope of the linear fitting equation is transdermal rate J [$\mu\text{g}/(\text{cm}^2 \text{ h})$], the time axis intercept is transdermal delay t_h .

$$Q_n = \frac{C_n V + \sum_{i=1}^{n-1} C_i V_s}{S} \quad (1)$$

where C_n and C_i in the equation above stand for the concentration ($\mu\text{g}/\text{mL}$) of receiving liquid detected at the n th and i th time, respectively, while V_s and V stand for the sampling volume (mL), medium volume (mL) and effective contact area (cm²), respectively¹⁴.

Determination of main components in extract of daphnes giraldii cortex in receiving liquid by HPLC method

Chromatographic conditions: HPLC conditions were as follows: PRODIGY ODS (3) (250 mm $\times$ 4.60 mm, 5 μm) column with temperature of 35 °C, mobile phase was methanol-0.05 % phosphate (18:82, 1 mL/min), detection wavelength was 327 nm and injection amount was 10 μL .

Sample preparation: Transdermal liquid sample: 1 mL receiving fluid was diluted to 2 mL with 50 % methanol.

Standard solution: 10.57 mg daphnin, 9.22 mg daphnetin-8-O- β -D-glycoside and 6.05 mg daphnetin reference compound were dissolved to 50 mL with 50 % methanol as the standard solution. The concentration of daphnin, daphnetin-8-O- β -D-glycoside and daphnetin was 0.2114, 0.1844 and 0.121 mg/mL, respectively.

The daphnes giraldii cortex extract (30 mg) was diluted to 25 mL with 50 % methanol to prepare the test sample.

RESULTS AND DISCUSSION

The standard solutions (0.5, 1, 2, 3, 4, 5 and 6 mL) were diluted to 10 mL with 50 % methanol, respectively, then injected to chromatography. Standard calibration curves were established by plotting peak areas against the concentration of the standards. For daphnin in the concentration range of 10.57-126.84 $\mu\text{g}/\text{mL}$, the regression equation was $Y = 18.354X - 4.2972$ ($r = 0.9999$, where Y is the peak area and X is the concentration of the standard); for daphnetin-8-O- β -D-glucoside, 9.22-110.64 $\mu\text{g}/\text{mL}$, $Y = 23.044X - 1.1564$ ($r = 1.0000$) and for daphnetin 6.05-72.6 $\mu\text{g}/\text{mL}$, $Y = 43.265X - 12.046$ ($r = 0.9999$). The results showed that our method has a good linearity.

Specificity: 10 μL of the blank solution (methanol), standard solution and test sample were injected into chromatography, respectively. The results showed that the three major components and impurities were well separated with no interference (Fig. 1).

Precision: The test sample was continuously injected for 5 times. The RSD of daphnin, daphnetin-8-O- β -D-glucoside and daphnetin were 0.07, 0.27 and 0.12 %, respectively. This method showed a good precision (RSD < 2 %).

Fig. 1. Chromatograms

Stability: Standard solution and test sample were injected every 2 h. The RSD of daphnin, daphnetin-8-O-β-D-glucoside and daphnetin were 1.16, 1.08 and 1.15 %, respectively. The results showed that the standard solution and test sample were stable in 8 h at the room temperature (RSD < 2 %).

Reproducibility: Five parallel test samples were prepared, then samples were injected. The RSD of daphnin, daphnetin-8-O-β-D-glucoside and daphnetin were 0.98, 0.95 and 1.07 %, respectively. The results showed the reproducibility was fairly good (RSD < 2 %).

Recovery: To spike the appropriate amount of the standard solution into 15 mg *daphnes giraldii* cortex extract. Samples were processed and analyzed. The results showed a good recovery (Tables 1-3).

Transdermal characteristics of *daphnes giraldii* cortex

Enhancer dosage: There are many factors that affect the percutaneous absorption of drugs, while enhancer and its amount are the important ones¹⁵.

There are many types of permeation enhancers, they can mainly be divided into sulfoxides, pyrrolyl ketones, azone,

fatty acids and their esters, surfactants, alcohols, polyhydric alcohols, terpenes, ethylenically amines and amides, phospholipids, carbohydrates, amino acids, the macrocyclic compounds, organic solvent according to their chemical structure. In them azone, propylene glycol (PG), oleic acid, linoleic acid are more widely used.

Menthol, borneol, azone and their mixture are commonly used enhancers. In recent years, azone is a non-toxic and stable enhancer which has good performance of permeation enhancement for both lipophilic and hydrophilic drugs¹⁶, nowadays it is recognized as an excellent penetration enhancer, it has little toxicity to the skin with small dosage; menthol and borneol are similar to azone in the penetration enhancing mechanism, while menthol and borneol have certain pharmacological activity; propylene glycol is often used in combination with azone, oleic acid, lauryl alcohol sulfate, *etc.*, which have a significant role in transdermal penetration, but compared with azone, it requires a higher concentration. Similarly, oleic acid, lauryl alcohol sulfate, sodium salicylate, *etc.* having a certain penetration-facilitating effect, but due to the high-dose and damage to skin structure, they are not widely applied. Thus in

TABLE-1
RECOVERY OF DAPHNIN

No.	Sample weight (mg)	Sample content (mg)	Content found (mg)	Added content (mg)	Calculated content (mg)	Recovery (%)	$\bar{X}$ (%)	RSD (%)
1	15.31	3.42	6.904	3.434	3.484	101.46	100.32	0.98
2	15.55	3.474	6.936	3.434	3.462	100.82		
3	15.19	3.393	6.861	3.434	3.468	100.99		
4	15.65	3.496	6.938	3.434	3.441	100.20		
5	15.15	3.385	6.745	3.372	3.36	99.64		
6	15.41	3.443	6.774	3.372	3.331	98.78		

TABLE-2
RECOVERY OF DAPHNETIN-8-O-β-D-GLUCOSIDE

No.	Sample weight (mg)	Sample content (mg)	Content found (mg)	Added content (mg)	Calculated content (mg)	Recovery (%)	$\bar{X}$ (%)	RSD (%)
1	15.31	2.757	5.708	2.994	2.951	98.56	98.87	1.47
2	15.55	2.801	5.725	2.994	2.925	97.66		
3	15.19	2.736	5.674	2.994	2.938	98.13		
4	15.65	2.819	5.738	2.994	2.92	97.49		
5	15.15	2.729	5.555	2.802	2.827	100.86		
6	15.41	2.775	5.591	2.802	2.816	100.50		

TABLE-3
RECOVERY OF DAPHNETIN

No.	Sample weight (mg)	Sample content (mg)	Content found (mg)	Added content (mg)	Calculated content (mg)	Recovery (%)	$\bar{X}$ (%)	RSD (%)
1	15.31	2.061	4.063	1.966	2.002	101.81	100.79	1.143
2	15.55	2.093	4.074	1.966	1.981	100.73		
3	15.19	2.045	4.049	1.966	2.005	101.96		
4	15.65	2.106	4.095	1.966	1.988	101.11		
5	15.15	2.039	3.865	1.824	1.825	100.07		
6	15.41	2.074	3.878	1.824	1.804	98.92		

this research, azone was selected as the enhancer to investigate various constituents in our formulation through the skin.

It can affect the structure of corneum keratin cell, which enables the drug recorded in reference documentation^{17,18}. A series of babu plaster of daphnes giraldii cortex extract containing different contents of azone and 1-3 times of daphnes giraldii cortex extract amount¹⁹ were prepared, respectively. Then the transdermal characteristics of the three major active components were discussed. The relation curves between the time and accumulative penetration ratio of the components with different doses of azone are as in Fig. 2. The penetration rates were calculated by eqn. (1) and the results were shown in Table-4.

The results showed that the effect of azone on the penetration rate was 2 times > 1 time > 3 times with a significant difference between 2 times and 1 time, 3 times ($P < 0.05$) for daphnin and daphnetin-8-O- β -D-glucoside. But the effect was 1 time > 2 times > 3 times with no significant difference ($P > 0.05$) for daphnetin and the total penetration. Therefore the amount of azone was determined as 2 times of the daphnes giraldii cortex extract amount.

The result also showed that with a certain range of the azone concentration, the penetration ratio of index components increased with the increase of it and decreased with the increase of it out of the range. Daphnin, daphnetin-8-O- β -D-glucoside (8-substituted-glucoside), daphnetin (7-substituted-glucoside)

(a) Daphnin (b) Daphnetin-8-O- β -D-glucoside (c) Daphnetin (D) Total

Fig. 2. Penetration curves with different doses of azone

TABLE-4
PENETRATION RATES WITH DIFFERENT DOSES OF AZONE (n = 3)

Components	Azone content (times of drug)	Penetration rates ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Transdermal delays (h)	Fitting equations
Daphnin	0	1.8000	—	—
	1	3.4745	4.28	$Y = 3.4745X - 14.855$, $r = 0.9790$
	2	4.1043	4.22	$Y = 4.1043 X - 17.329$, $r = 0.9820$
	3	1.9769	3.97	$Y = 1.9769 X - 7.8408$, $r = 0.9843$
Daphnetin-8-O- β -D-glucoside	0	1.5000	—	—
	1	3.7918	4.25	$Y = 3.7918 X - 16.118$, $r = 0.9796$
	2	4.0429	4.24	$Y = 4.0429 X - 17.146$, $r = 0.9813$
	3	2.0239	3.96	$Y = 2.0239 X - 8.0148$, $r = 0.9848$
Daphnetin	0	1.6000	—	—
	1	6.1165	3.56	$Y = 6.1165 X - 21.765$, $r = 0.9925$
	2	4.0299	4.20	$Y = 4.0299 X - 16.915$, $r = 0.9826$
	3	2.9193	3.76	$Y = 2.9193 X - 10.985$, $r = 0.9892$
Total	0	4.9000	—	—
	1	13.7224	3.95	$Y = 13.7224 X - 54.243$, $r = 0.9859$
	2	13.0799	4.22	$Y = 13.0799 X - 55.203$, $r = 0.9820$
	3	6.2421	3.88	$Y = 6.2421 X - 24.196$, $r = 0.9867$

with different polarity, molecular weight and groups have different permeation rate increase with different concentration of enhancer, they are 92.8, 152.7 and 282.5 % (1 time); 128, 169.5 and 151.9 % (2 times); 9.8, 34.9 and 82.5 % (3 times) respectively.

Drug loadings: A series of babu plaster of *daphnes giraldii* cortex extract were prepared with loading different contents of drug: 2, 5 and 10 times of the clinical dosage, respectively. We studied the transdermal characteristics of the three major active components through rabbit skin. The relation curves

between the accumulative penetration rate of the components with different drug loadings and time are as in Fig. 3.

The penetration rates were calculated by eqn. (1) and the results were shown in Table-5. The results revealed that the permeation rates and transdermal delays of the three different loadings were similar. There were no significant differences among the three series of babu plaster of *daphnes giraldii* cortex extract ($P > 0.05$). Therefore the clinical dose (2 times) is determined as the optimum drug loading for the transdermal delivery system.

(a) Daphnin (b) Daphnetin-8-O- β -D-glucoside (c) Daphnetin (D) Total

Fig. 3. Penetration curves with different drug loadings

TABLE-5
PENETRATION RATES WITH DIFFERENT DOSES OF AZONE (n = 3)

Components	Azone content (times of drug)	Penetration rates ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Transdermal delays (h)	Fitting equations
Daphnin	2	5.2756	4.2014	$Y = 5.2756X - 22.165, r = 0.9772$
	5	4.9390	4.0723	$Y = 4.9390X - 20.113, r = 0.9802$
	10	5.5882	4.3671	$Y = 5.5882X - 24.404, r = 0.9802$
Daphnetin-8-O- β -D-glucoside	2	5.0795	4.0982	$Y = 5.0795X - 20.817, r = 0.9805$
	5	4.6654	3.9917	$Y = 4.6654X - 18.623, r = 0.9815$
	10	5.3252	4.3710	$Y = 5.3252X - 23.276, r = 0.9767$
Daphnetin	2	4.6079	3.7289	$Y = 4.6079X - 17.182, r = 0.9882$
	5	5.1240	3.9848	$Y = 5.1240X - 23.561, r = 0.9844$
	10	5.9043	4.1681	$Y = 5.9043X - 24.61, r = 0.9816$
Total	2	14.9276	4.0627	$Y = 14.9276X - 60.645, r = 0.9811$
	5	15.3181	4.0182	$Y = 15.3181X - 61.55, r = 0.9821$
	10	16.6555	4.3080	$Y = 16.6555X - 71.754, r = 0.9784$

Conclusion

In this study, we have developed a method for determining the three effective components in the extract of daphnes giraldii cortex simultaneously. The effect of drug loadings and permeation enhancer was also investigated on the transdermal characteristics of the components. The results indicate that the active components in the extract of daphnes giraldii cortex have the high percutaneous abilities, so it is suitable for the transdermal delivery system.

ACKNOWLEDGEMENTS

This study was supported by Key Science-technology Plan Project of Tianjin "Research on Daphnes Giraldii Cortex and its gel"(12ZCDZSY12000). The authors are also grateful to the Traditional Chinese Medicine Department of Tianjin Institute of Pharmaceutical Research for providing the experimental apparatus.

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