AAPS PharmSciTech, Vol. 12, No. 4, December 2011 (© 2011)
DOI: 10.1208/s12249-011-9672-4

Research Article

Theme: Advances in Pharmaceutical Excipients Research and Use: Novel Materials, Functionalities and Testing
Guest Editors: Otilia Koo, Thomas Farrell, Allison Radwick, and Sameer Late

Enhanced Intestinal Absorption of Daidzein by Borneol/Menthol Eutectic Mixture and Microemulsion

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Received 16 August 2010; accepted 4 August 2011; published online 13 August 2011

Abstract. In the present study, the effect of a borneol/menthol eutectic mixture (25:75) and microemulsion on the absorption of daidzein in rat intestinal membrane was evaluated. The microemulsion formulation was composed of ethyl oleate (oil), Cremophor RH40 (surfactant), PEG400 (co-surfactant), and water. The borneol/menthol eutectic mixture and its microemulsion were found to enhance the intestinal absorption of daidzein *in vitro*. A diffusion chamber system with isolated rat intestinal membranes was used. In contrast, verapamil (0.3 mM), a typical P-glycoprotein inhibitor, showed no effect on the absorption of daidzein by this system. A pharmacokinetic study was conducted in rats. After oral administration of daidzein at a dose of 10 mg/kg in the form of either borneol/menthol eutectic mixtures or suspension, the relative bioavailability of borneol/menthol eutectic mixtures and microemulsion was enhanced by about 1.5- and 3.65-fold, respectively, compared with a daidzein suspension. In conclusion, a borneol/menthol eutectic mixture can enhance the absorption of daidzein, although the mechanism of absorption enhancement is still unclear.

KEY WORDS: absorption enhancer; bioavailability; borneol/menthol eutectic mixture; daidzein; microemulsion.

INTRODUCTION

Oral drug administration is preferred because of its convenience and better compliance of patients. However, the development of oral delivery systems is challenging. Some drugs have poor water solubility and permeability, as well as rapid metabolism. A variety of formulation strategies have been developed to improve the solubility and bioavailability of such drugs. These strategies include using absorption enhancers (1,2), chemical modification (3,4), and different dosage forms (*e.g.*, microemulsion) (5,6). The use of absorption enhancers is the most common. Various kinds of intestinal absorption enhancers include surfactants, bile salts, chelating agents, and fatty acids (1,7). Some studies have also shown that borneol and menthol improve the oral bioavailability of some drugs (8,9). A borneol/menthol eutectic mixture has enhanced the brain transport of a drug when it was nasally coadministered with a polypeptide molecule (10). Daidzein (also called 4′,7-dihydroxylisoflavone) (Fig. 1), a water-insoluble isoflavone, is mainly present in leguminous plants, especially in soybeans, soy foods, and *Pueraria lobata* Ohwi (Leguminosae) (11,12). Daidzein can reduce breast cancer occurrence and also plays a key role in protecting against colon cancer (13). More recently, Wilcox and Blumenthal (14,15) have hypothesized that isoflavones may reduce the aggregation of platelets at the sites of arterial injury. These sites are associated with atherosclerotic development.

Nevertheless, the beneficial effects of bioactive compounds depend on their bioavailability and on intestinal absorption. Studies on the absorption, metabolism, and distribution of isoflavones are limited (16–18). A study on isoflavone bioavailability in healthy women has shown a curvilinear relationship between dietary intake and plasma levels (19).

In the present study, attention was focused on the intestinal absorption characteristics of daidzein because data regarding the absorption of this isoflavone are very rare. The effects of verapamil on the daidzein transport across rat intestinal membrane were examined. The influences of a borneol/menthol eutectic mixture and its microemulsion on the intestinal absorption and oral bioavailability of daidzein were also investigated.

EXPERIMENTAL

Materials and Methods

Chemicals and Reagents

Diethyl ether, polyethylene glycol 400 (PEG400), borneol, and menthol were purchased from the Shanghai Chemical

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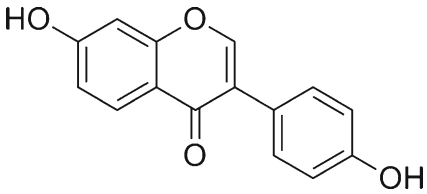

Fig. 1. Structure of daidzein

Reagents Institute (Shanghai, People's Republic of China). Verapamil was supplied by the Shanghai Xinyi Pharmaceutical Co. Ltd. (Shanghai, People's Republic of China). Daidzein was provided by the Qingze Co. Ltd. (Nanjing, People's Republic of China). The purity of these reagents and drugs was 98%, as verified by high-performance liquid chromatography (HPLC).

Apparatus and Chromatographic Conditions

The HPLC analysis was conducted on a Waters (Waters Corp., MA, USA) equipped with 1,525 controller pumps and 2,487 detectors and configured with the Millennium 3.2 software. Samples were loaded into the column using a 717-plus autosampler (Waters Corp., MA, USA). The analytical column was a reversed-phase C_{18} column (150×4.6 mm i.d.; particle size=5 μm; ELITE, Dalian, People's Republic of China). The mobile phase was methanol in distilled deionized water (50:50, *v*/*v*), and the flow rate was 1.0 mL/min. The analytes were detected by a UV detector at 260 nm.

Solution Preparation

The stock solution of daidzein (1 mg/mL) was prepared in methanol and stored at 4°C. The plasma samples were stored at −20°C until use. The working solutions were prepared on the day of analysis by further dilution of the stock solutions with methanol. A borneol/menthol eutectic mixture (25:75) was prepared by triturating in a mortar and pestle for 1 min.

Animals

Healthy male Sprague–Dawley rats (200–250 g) were purchased from the Animal Resources Center, Shanghai Jiaotong University, Shanghai, People's Republic of China. The rats were kept in a room under controlled temperature (22±1°C) and an automatic day–night rhythm (12-h cycles). The rats were housed on wire-bottom cages underlined with paper. All experiments were approved by the Ethical Committee of the Shanghai Jiao Tong University, Shanghai, People's Republic of China. The rats were provided with free access to tap water and food. After overnight fasting, the animals were anesthetized with diethyl ether. The intestine was exposed through a midline abdominal incision, removed, and washed in ice-cold saline. Intestinal segments, excluding Peyer's patches, were isolated and immersed in an ice-cold Tris–4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.4) containing 25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 1 M Tris solution.

Solubility Studies

Excess daidzein was added to water, acidic water, and borneol/menthol eutectic mixture using an ultrasonic cleaner at 40°C in a water bath for 20 min. The mixture was then shaken at 25°C for 48 h. The samples were filtered by a 0.45-μm membrane filter. The filtrate was diluted with methanol, and the concentration of daidzein was determined by HPLC.

Microemulsion Preparation

The microemulsion was prepared using a method we previously reported (20). Given that daidzein was difficult to dissolve, daidzein and the borneol/menthol eutectic mixture was first dissolved by a co-surfactant. The oil and surfactant were slowly added with gentle stirring until a homogeneous mixture was formed. Water was also slowly added dropwise under 600 rpm of magnetic stirring at 37°C. The oil phase was ethyl oleate, the surfactant was Cremophor RH40, and the co-surfactant was PEG400. The optimal microemulsion was composed of 6% ethyl oleate, 20% Cremophor RH40, 10% PEG400, and 60% aqueous phase (*w*/*w*).

Absorption Experiment

The absorption of daidzein in the rat intestinal membrane was studied with a diffusion chamber system (Corning Coster Corp.) (21–23). The diffusion cells had a surface area of 1.78 cm^2 and a volume of 7 mL. The intestinal segments were cut open, and the intestinal sheets were mounted onto the pins of the cell. The half-cells were clamped together. Daidzein solutions were freshly prepared by dissolving in dimethyl sulfoxide (DMSO). They were then diluted with a Tris–HEPES buffer solution to yield a final concentration of 20 μg/mL. The final concentration of DMSO in the solution was 1% (*v*/*v*). The concentrations of the excipients during the *in vitro* permeation experiments were 0.1–3% borneol/menthol (25:75) eutectic mixtures. The borneol/menthol eutectic mixtures concentration in the microemulsion was 3%. A drug solution (7 mL) was added to the donor site, whereas the same volume of drug-free Tris–HEPES buffer solution was added to the opposite site. The temperature of the intestinal membranes was maintained at 37°C, and both fluids were circulated by a gas lift with 95% O_2/5% CO_2. During the absorption studies, aliquots were taken from the receptor chamber at predetermined times of up to ~2 h. The receptor chamber samples were replaced with an equal

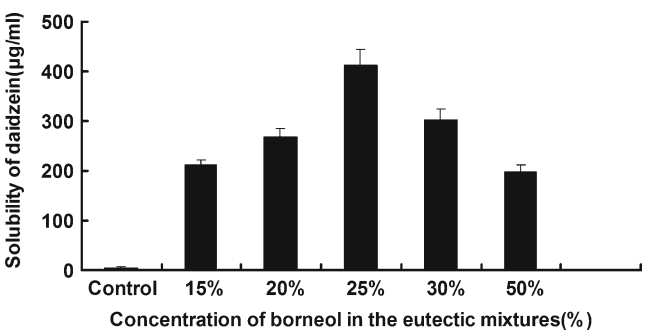

Fig. 2. Solubility of daidzein in various concentrations of borneol in a eutectic mixture

Table I. Effects of Verapamil on the Permeability of Daidzein Across the Intestinal Membranes

Drug	Excipient	P_{app}($\times10^{-6}$ cm/s)		Ratio (P_{appsm}/P_{appms})
		M to S	S to M	
Daidzein (8 μg/mL)	Control	17.11±2.32	22.25±2.83	1.30
	0.3 mM verapamil	18.62±2.13 N.S.	22.68±2.66 N.S.	1.22 NS
Daidzein (20 μg/mL)	Control	8.83±1.21	12.13±1.62	1.37
	0.3 mM verapamil	8.32±1.12 N.S.	11.02±1.47 N.S.	1.32 NS

Each value represents the mean±SD of at least three experiments
NS not significantly different, *M to S* mucosal to serosal, *S to M* serosal to mucosal

volume of Tris–HEPES buffer solution, and the permeated drugs were assayed. The apparent permeability coefficients (P_{app}) of the drug were calculated from the slope of the linear portion of the drug permeability-time profiles by the relationship $P_{app}=(dX_R/dt)\times(1/A\cdot C_0)$, where P_{app} is the apparent permeability coefficient, X_R is the amount of drug in the receptor side, A is the diffusion area, and C_0 is the initial drug concentration in the donor side. The efflux ratio was used to evaluate the extent of efflux (24–27).

The viability of the intestinal membrane during the test period was monitored by measuring trypan blue dye transport and electrophysiological parameters. There was no dye transport and no remarkable change in the electrophysiological parameters. This finding confirmed that the viability of the intestinal membrane was maintained during the transport experiments.

Pharmacokinetic Studies

Male Sprague–Dawley rats were fasted overnight with free access to water before drug administration. Daidzein was suspended in a 0.5% sodium carboxymethylcellulose solution as the control sample. Subsequently, 3% borneol/menthol eutectic mixtures were added to the daidzein suspension. The borneol/menthol eutectic mixture microemulsion was orally administered via the esophagus into the stomach (10 mg/kg of rat) in 2 mL using a blunt needle. Blood samples (200 μl) were collected into heparinized micro-centrifuge tubes (100 IU/mL blood) from the caudal vein at designated times. The blood samples were centrifuged for 5 min at 5,000×*g*, and 100 μl of plasma was mixed with 200 μl of acetonitrile in an Eppendorf tube. The tubes were vortexed for 1 min and centrifuged at 5,000×*g* for 10 min. Afterwards, 20 μl of the resulting supernatant was injected into the HPLC system. The area under the plasma concentration-time curve from time zero to the final sampling time (12 h) was calculated by the linear trapezoidal rule. The peak plasma concentration (C_{max}) and the time to reach it (T_{max}) were obtained from the experimental data.

Statistical Analysis

Statistical evaluations were performed by Student's *t* tests of the paired observations to analyze the different concentrations of daidzein. *P* values of <0.05 were considered to indicate significant differences. Data are expressed as the mean±SD of at least three experiments.

RESULTS

Solubility Studies

The actual solubilities of daidzein are 8.215 μg/mL (in water) and 7.853 μg/mL (in acidic water) (20). The solubility of daidzein in various borneol/menthol eutectic mixtures is presented in Fig. 2. The solubility of daidzein increased with various concentrations of borneol in the eutectic mixture. The highest solubility was observed at the borneol/menthol ratio of 25:75. Therefore, 25:75 was selected as the ratio of the borneol/menthol eutectic mixture.

Effects of Verapamil on Daidzein Transport Across the Intestinal Membrane

Table I shows the effects of verapamil on daidzein transport across rat intestinal membrane. The absorptive or secretory transport of daidzein had no significant difference in

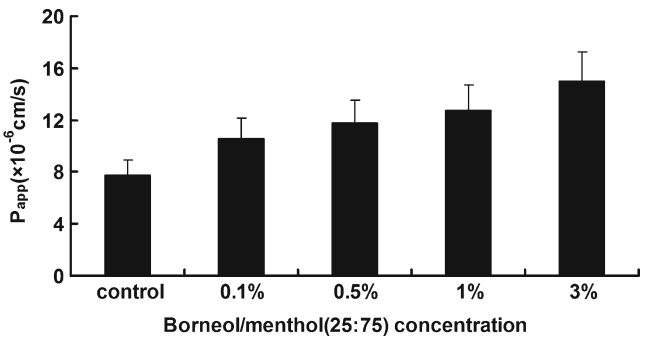

Fig. 3. Effects of a borneol/menthol eutectic mixture on the absorption of daidzein in rat intestinal membrane. Data are the mean±SD of at least three determinations

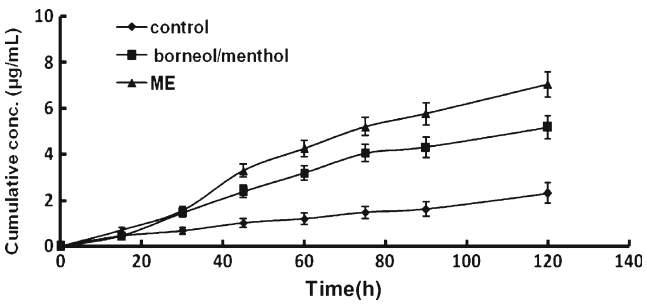

Fig. 4. Time course of daidzein absorption (*diamonds*) as well as the effects of a borneol/menthol (25:75) eutectic mixture (3%; *squares*) and a borneol/menthol (3%) microemulsion (*triangles*) on daidzein absorption in rat intestinal membrane. Data are the mean±SD of at least three determinations

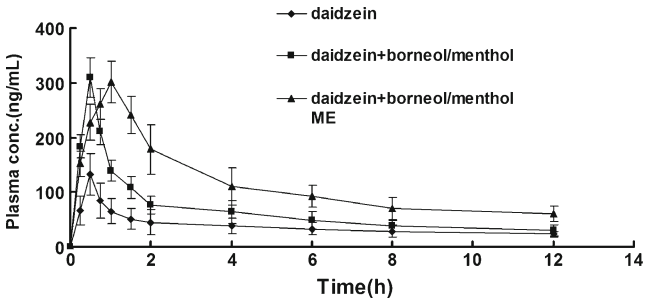

Fig. 5. Plasma concentration-time profiles of daidzein in rats after oral administration (10 mg/mL) of daidzein alone (*diamonds*) as well as in combination with borneol/menthol eutectic mixtures (*squares*) and borneol/menthol microemulsion (*ME*; *triangles*). Data are expressed as the mean±SD of at least three experiments

the presence of 0.3 mM verapamil, which is a typical P-glycoprotein (P-gp) inhibitor. The transport direction of daidzein and the function of P-gp were also evaluated by calculating the efflux ratio (P_{appsm}/P_{appms}) of daidzein. The efflux ratio of daidzein had no significant difference in the presence of verapamil.

Effects of Borneol/Menthol Eutectic Mixture and Microemulsion on the Absorption of Daidzein in Rat Intestinal Membrane

Figure 3 shows the effect of various concentrations of borneol/menthol (25:75) eutectic mixtures on the absorption of daidzein in rat intestinal membrane. When daidzein was added to 0.1–3% borneol/menthol (25:75) eutectic mixture solution, the absorption P_{app} significantly increased compared with the control group.

Figure 4 shows the time course of daidzein absorption (mucosal to serosal) in rat intestinal membrane. When 3% borneol/menthol (25:75) eutectic mixtures and microemulsion (containing 3% borneol/menthol eutectic mixtures) were added to the mucosal side, the absorption of daidzein significantly increased. The absorption trend of the formulations was as follow: borneol/menthol microemulsion>borneol/menthol eutectic mixtures>control group (daidzein solution).

Pharmacokinetic Studies

The plasma concentration-time profiles of daidzein (10 mg/kg) given alone and in combination with 3% borneol/menthol eutectic mixtures or microemulsion are given in Fig. 5. Daidzein plasma concentration significantly increased after oral administration of daidzein with borneol/menthol eutectic mixtures and microemulsion.

Table II shows the pharmacokinetic parameters of daidzein after it was administrated alone and in combination with borneol/menthol eutectic mixtures or borneol/menthol eutectic mixtures microemulsion. C_{max} of daidzein administered with borneol/menthol eutectic mixtures and microemulsion was 2.3-fold higher than that with daidzein alone. Compared with the daidzein suspension, the relative bioavailability of daidzein administered with borneol/menthol eutectic mixtures was 1.5-fold higher, whereas that of the borneol/menthol eutectic mixtures microemulsion was 3.65-fold higher. The borneol/menthol eutectic mixtures and microemulsion had a greatly enhancing effect in the *in vivo* study of daidzein.

DISCUSSION

Preparations and nutritional supplements containing daidzein are widely consumed for their potential health effects. However, the oral bioavailability of daidzein is very poor. In rats, the absolute bioavailability of daidzein was found to be 12.2%, which limits its curative effect (28–31).

In the present study, we investigated the effects of a P-gp inhibitor and absorption enhancer on daidzein transport across rat intestinal membrane. The purpose was to enhance the bioavailability of daidzein.

Many drugs are well absorbed by the gastrointestinal tract. The small intestine is the principal site of drug absorption. Typical *in vitro* experiments (or predicted values) include measuring drug permeability in intestinal tissue. However, the intestinal absorption properties of the major ingredients of most traditional Chinese medicines are unknown. In the present study, an *in vitro* diffusion chamber system was chosen, and isolated rat intestinal membranes were used as the intestinal transport model.

The absorptive or secretory transport of daidzein had no significant difference in the presence of 0.3 mM verapamil, a typical P-gp inhibitor. At the 8 μg/mL of daidzein, the P_{app} of daidzein was $17.11\pm2.32\times10^{-6}$ cm/s, it was similar to 21.26×10^{-6} cm/s reported by Ge (32). But at 20 μg/mL of daidzein, the P_{app} of daidzein in both directions decreased, a possible reason is consistent with incomplete solubility at the concentration. The most of daidzein was attached to intestine wall or sank to the bottom. Likewise, the efflux ratio of daidzein had no significant difference in the presence of verapamil. These results implied that P-gp may not be involved in the efflux of daidzein.

Table II. Pharmacokinetic Parameters of Daidzein after Oral (10 mg/kg) Administration in Rats in the Presence of Borneol/Menthol Eutectic Mixtures and Microemulsion (ME)

Administrations	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-12} (ng h^{-1} mL^{-1})
Daidzein only	132.52±29.61	0.43±0.05	371.36±102.17
Daidzein+borneol/menthol	309.78±32.92*	0.41±0.03 N.S.	561.03±158.48*
Daidzein+borneol/menthol ME	300.81±38.46*	1.0±0.16*	1,355.87±202.23**

Each value represents the mean±SD of at least three experiments
NS not significantly different
*$p<0.05$, compared with control; **$p<0.01$, compared with control

Solubility and permeability are the fundamental determinants of the oral bioavailability of a drug (33). From Fig. 3, borneol/menthol eutectic mixtures can increased the absorption P_{app}, one reason is eutectic mixtures could increase the solubility of daidzein. Absorption-promoting systems are extensively used to increase drug absorption by the intestine. These systems enable increased solubility of hydrophobic drugs, increased membrane fluidity or disruption of tight junctions, interaction with metabolic enzymes, and inhibition of efflux transporters (34,35). Borneol and menthol are extensively used separately as mucosa absorption enhancers (8,36). A borneol/menthol eutectic mixture, nasally administered with a polypeptide molecule, significantly enhanced the brain transport of the drug (10). However, few reports exist on the use of borneol/menthol eutectic mixtures as intestinal absorption enhancers for increasing the oral absorption of drugs. Therefore, these mixtures were applied in the present study to improve the intestinal absorption of daidzein. The results indicated that these mixtures enhanced the bioavailability of daidzein by improving its solubility and permeability. A microemulsion is a lipid-based delivery system that has the advantages of high solubilization, thermodynamic stability, and surfactant-induced permeability enhancement (37,38). A microemulsion system is considered as an ideal alternative for the oral delivery of some drugs, such as nitrendipine, ibuprofen, and docetaxel. Therefore, employing a microemulsion formulation can improve oral bioavailability (5,6,39). Compared with a daidzein-borneol/menthol eutectic mixture, a daidzein-borneol/menthol microemulsion is a formulation that contains a surfactant (Cremophor RH40, PEG400). The surfactant performs an absorption promotion effect. Hence, a microemulsion combined with a eutectic mixture may result in synergism. A borneol/menthol microemulsion can thus largely improve bioavailability compared with a borneol/menthol eutectic mixture. In the present study, T_{max} was determined from experimental measurements because the mechanism of absorption enhancement is still unclear. This is also the reason why T_{max} was speculated as responsible for drug release from a microemulsion, and for the increase of drug absorption. According to previous studies, many formulations behave similarly (40–42).

A borneol/menthol mixture is often used as a traditional Chinese medicines, has also been reported as safe for clinical practice (43). The therapeutic potential of this mixture and its possible application as a pharmaceutical excipient warrants further investigations.

CONCLUSIONS

A borneol/menthol eutectic mixture improved the bioavailability of daidzein by enhancing its solubility and permeability. A borneol/menthol eutectic mixture microemulsion enabled a higher bioavailability of daidzein compared with the control group. The results indicated the potential use of borneol/menthol eutectic mixtures for enhancing the absorption of poorly water-soluble drugs.

ACKNOWLEDGMENT

The present work was supported by the Shanghai Municipal Committee of Science and Technology (grant 08DZ1971304), the National Basic Research Program of China (973 Program; no. 2007CB936004), as well as the Key Projects of the National Innovative Drug R&D (2009ZX09502-009).

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