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Preparation of icariside II-phospholipid complex and its absorption across Caco-2 cell monolayers

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In the present study, an icariside II-phospholipid complex was prepared, and its physicochemical properties including UV spectrum, IR spectrum, differential scanning calorimetry (DSC) were tested. Furthermore, the absorption of icariside II and icariside II–phospholipid complex was compared in a Caco-2 cell culture model. The results show that icariside II–phospholipid complex could significantly increase the A-B transport of icariside II ($P < 0.01$), with A-B Papp of $(3.92 \pm 0.50) \times 10^{-6}$cm/s compared to control $(2.05 \pm 0.18) \times 10^{-6}$cm/s (increased by 91%). Likewise, the B-A transport of icariside II was significantly enhanced ($P < 0.01$), with Papp of $(15.3 \pm 0.72) \times 10^{-6}$cm/s (control) to $(22.4 \pm 1.4) \times 10^{-6}$cm/s (46% increase). Efflux ratio of icariside II decreased by 23.5% after forming icariside II–phospholipid complex. The therefore, it could be concluded that phospholipid complexation can increase the intestinal absorption of icariside II.

1. Introduction

Herba Epimedii, a herb derived from the dried aerial part of *Epimedium sagittatum Maxim*, has traditionally been used as a tonic, aphrodisiac and antirheumatic drug in China for many years (China Pharmacopoeia Committee 2010). The main active constituents of Herba Epimedii are flavonoids (Ma et al. 2011), such as icariin and icariside II (Fig. 1). Icariside II, a metabolite of icariin, is a flavonoid glycoside (Xu et al. 2006). It has been reported that icariside II can induce apoptosis in human PC-3 prostate cancer cells (Lee et al. 2009) and inhibit hypoxia-inducible factor-1 alpha in U266 multiple myeloma cells (Kim et al. 2011). It can also significantly improve ALP activity and enhance the gene expression of bFGF, IGF-1, Osterix and Runx-2 (Zhai et al. 2010). However, low bioavailability of icariside II limits its efficacy in humans (Jeong et al. 2005).

Therefore, it is of great importance to develop an easy method to improve the absorption and the therapeutic effect of icariside II. Phospholipids are an important component of cell membranes which are responsible for cell membrane fluidity and make drugs easily be absorbed (Xiao et al. 2006). Preparing a phospholipid complex is a common method to increase bioavailability of drugs (Chen et al. 2010). It was reported that a phospholipid complex can improve the gastrointestinal absorption to acquire a higher drug concentration, and the technique is convenient to be practiced. Several natural drugs, such as silymarin (Morazzoni et al. 1993), clarithromycin (Yan et al. 2009), and curcumin (Kuntal et al. 2007), have been found to become better bioavailable in phospholipid complex formulations. The Caco-2 cell culture model is routinely used to investigate drug absorption *in vitro* and is recognized by the FDA as a viable model of human intestinal absorption. (Artursson et al. 1991; Boulenc et al. 1993; Karamustafa et al. 2009).

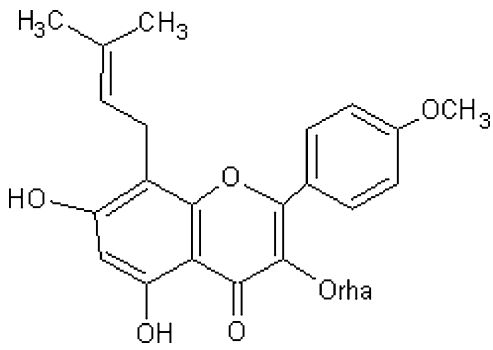

Fig. 1: Chemical structure of icariside II

However, few papers have described the Caco-2 cell culture model to evaluate the absorption on phospholipid complexes (Mutlu et al. 2008), and to the best of our knowledge, no paper has been found about Caco-2 cell culture model being used for test absorption and excretion enhancement of phospholipid complex to flavonoids. In this present study, a phospholipid complex of icariside II was prepared and its physicochemical properties life determined. Absorption and excretion of icariside II were compared between the free icariside II and its phospholipid complex.

2. Investigations and results

Transport of icariside II (10 μM) and complex (contain icariside II 10 μM) across Caco-2 monolayers were examined for 4 h in

A–B (absorptive) and B–A (secretory) directions. We found that the icariside II–phospholipid complex can promote the absorption and advance the secretory at the same time. The complex would promose a better absorption than icariside II in the Caco-2 cell culture model.

2.1. Determination of icariside II content in the complex

The content of icariside II–phospholipid complex is 8.0 mg icariside II–phospholipid complex includes 3.0 mg icariside II (i.C. % 37.5%). The icariside II content in phospholipids complex (i.C. %) was calculated as follows: i.C. % = (icariside II amount/ phospholipids complex weight) × 100.

2.2. UV spectrum

Icariside II and icariside II–phospholipid complex were dissolved in methanol. In HPLC analysis, the DAD acquisition wavelength was set in the range of 200–400 nm. Retention times and UV absorption of icariside II and icariside II–phospholipid complex were the same. So we can infer that the structure of icariside II in the complex did not change and icariside II–phospholipid complex was formed instead of a new compound.

2.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry is a rapid and reliable way to screen icariside II and phospholipids compatibility and it provides maximum absorption about possible interactions. Fig. 2 shows the DSC curves of icariside II, phospholipids, physical mixture and icariside II–phospholipid complex. The thermogram of icariside II exhibited three different peaks: first peak (167.2 °C), second one (195.9 °C) and the third one (218.9 °C) might be caused by the partial schizolysis of icariside II. However, phospholipids exhibited two clearly different kinds of endothermal peaks. The first endothermal peak at 126.4 °C was mild, which suggested that the formation of this peak was due to hot movements of the polarpart of the phospholipid molecule. Moreover, the second sharp endothermal peak (365.7 °C) could be formed owing to the phase transition from a gel-like state to a liquid crystal state, and the carbon-chain in the phospholipids may have perhaps undergone melting, isomeric or crystal changes (Maiti et al. 2007). Then, there is also a exothermic peak (327.9 °C) existing. The physical mixture of icariside II and phospholipids shows several peaks. The former has the same onset temperature (165.1 °C) of icariside II and the latter has the same onset temperature (391.8 °C) than the complex. It may be assumed that with the rise in temperature the phospholipids become melted and icariside II got dissolved in phospholipids, partly forming the complex (Parmar et al. 2011; Xiao et al. 2006). The lastest has the temperature (262.4 °C) of phospholipids. DSC of phospholipid complex shows that the endothermal peaks of drug had disappeared, but the absorption of phospholipids are existing. There is a new peak (391.8 °C) formed by icariside II and phospholipids through some interaction such as the combination of hydrogen bonds or van der Waals forces.

2.4. IR spectra analysis

The IR spectra of phospholipids, icariside II, physical mixture and phospholipid complex are shown in Fig. 3. It was obvious that the physical mixture and the complex indicated distinct IR spectra. Compared with the complex, the peaks at 1738 cm^{-1} (due to phospholipid OH–C=O) and 1650 cm^{-1} (due icariside II C=O) were obviously found in the spectrum of the physical mixture. However, in the spectrum of their complex, the characteristic absorption peak of icariside II was almost masked. Moreover, no new peaks were observed in the mixture and complex. This indicates indicates that it was the phospholipid complex instead of a new compound or physical mixture.

Table 1: Permeabilities (Papp) and efflux ratios of icariside II and icariside II–phospholipid complex Absorptive permeability was expressed as A-B, whereas secretory permeability was expressed as B-A. Data are expressed as mean ± SD (n = 3). Efflux radio was Papp(B-A)/Papp(A-B)

Compound concentration	Papp($\times 10^{-6}$ cm/s) A–B	B–A	Efflux ratio
incariside II (10 μM)	2.05 ± 0.18	15.3 ± 0.72	7.46
icariside II-phospholip id complex (10 μM incariside II)	3.92 ± 0.50	22.4 ± 1.4	5.71

2.5. Transport of icariside II and icariside II–phospholipid complex across Caco-2 cell monolayers

Icariside II and icariside II–phospholipid complex absorbtion across Caco-2 cells is shown in Fig. 4, which is linear with time, demonstrated with the time variation. At the same time, also excretion of icariside II exists. And excretion is apparently larger than absorption. From the icariside II–phospholipid complex, Caco-2 cell monolayers increase absorption and promote excretion simultaneously. This may be due to more easily binding with the cell membrane after the formation of complex not only for A–B (absorptive) directions, but also for B–A (secretory) directions. As illustrated in Fig. 5, the facilitative effects of absorption are noticeable with the icariside II–phospholipid complex, suggesting no saturation of cellular accumulation. Icariside II 10 μM and icariside II–phospholipid complex (contain icariside II 10 μM) exhibited a bi-directional transport and apparent permeability coefficients (Papp) both absorptive and secretory directions as shown in the Table. Icariside II was transported in the secretory (B–A) direction at a higher rate than in the absorptive (A–B) direction. The basolateral to apical (B–A) efflux of icariside II was markedly greater than its apical to basolateral (A–B) flux, indicating a significant efflux of this compound. Icariside II–phospholipid complex significantly ($P < 0.01$) increased the A-B transport of icariside II; A-B Papp of $(3.92 \pm 0.5) \times 10^{-6}$cm/s compared to control $(2.05 \pm 0.18) \times 10^{-6}$cm/s (91% increase). Likewise, the B-A transport of icariside II was significantly ($P < 0.01$) enhanced, Papp of $(15.3 \pm 0.72) \times 10^{-6}$cm/s (control) to $(22.4 \pm 1.4) \times 10^{-6}$cm/s (46% increase). Efflux ratios of icariside II and icariside II–phospholipid complex were 7.46 and 5.71, respectively. At the end of the transport experiment, integrity of the monolayer was monitored by TEER value determination, and there was no significant change. These results indicated that phospholipid complexation can enhance absorption of icariside II.

3. Disscusion

Some studies have indicated the beneficial role of phospholipids in enhancing the therapeutic efficacy of some molecules showing poor oral absorption. Natural active ingredients and the phospholipid can be complexed under certain conditions. The complex can effectively enhance the absorption of the natural active ingredients *in vivo*, dramatically improve its bioavailability and have stronger pharmacological effects. It was observed

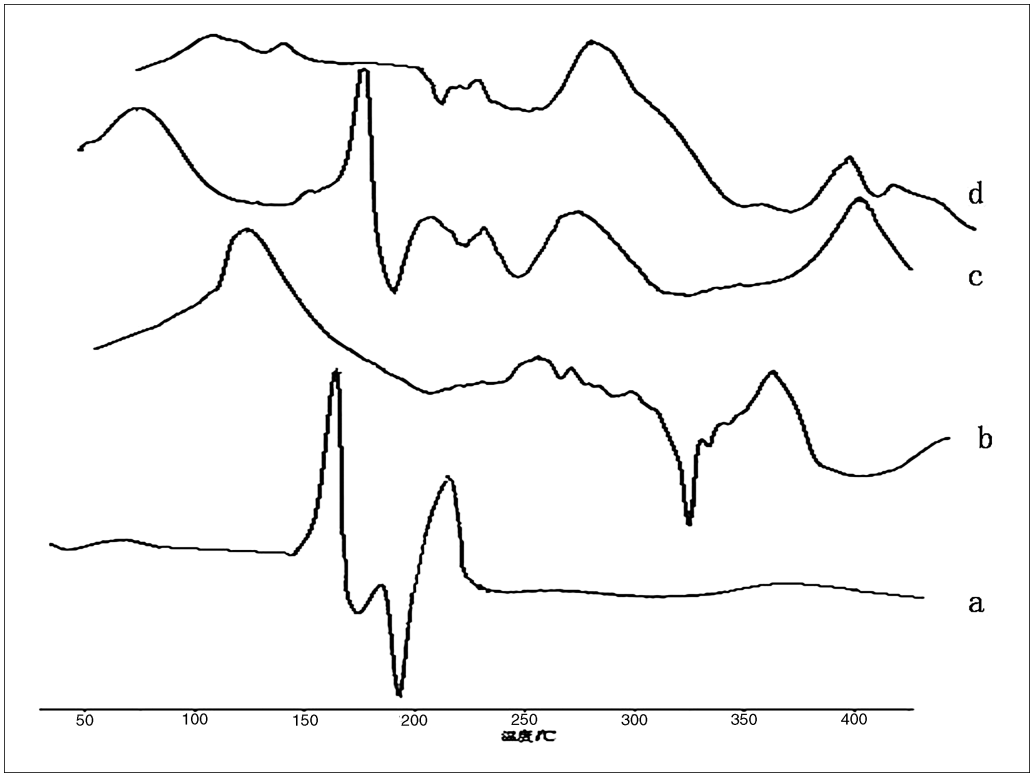

Fig. 2: DSC thermograms of icariside II (a), phospholipids (b), physical mixture (c) and icariside II–phospholipid complex (d)

that the phospholipid complex has significant upper hand over the pure molecule in exerting activities (Comoglio et al. 1995; Carini et al. 1992; Conti et al. 1992; Morazzoni et al. 1993). That may be because after formation phospholipid complex, the drugs are more expediently combined with the cell membrane which consisted of phospholipids.

Icariside II has shown potential anti-cancer activity (Choi et al. 2008; Lee et al. 2009). However, icariside II is poorly bioavailable because it is extensively effluxed by the apically located efflux transporters, which restrict effectiveness of icariside II (Chen et al. 2008). So we tried to improve the efficacy of icariside II by preparing a phospholipid complex, which may increase the absorption of icariside II.

Caco-2 cell models have many advantages when compared with classical drug absorption models (Audus et al. 1990); rapid estimation of the permeation or metabolism of drugs, opportunity for studying a drug absorption mechanism under controlled conditions, rapid estimation of increasing drug absorption methods by using prodrugs, absorption enhancers or other pharmaceutical materials. But, has rarely been used in evaluating intestinal absorption of phospholipid complexes. Here it was used to evaluate the absorption of icariside II–phospholipid complex firstly. In this protocol, we successfully prepared icariside II–phospholipid complex by a simple method. UV spectrum shows that the structure of icariside II in the complex did not change. DSC curves of phospholipids complex showed

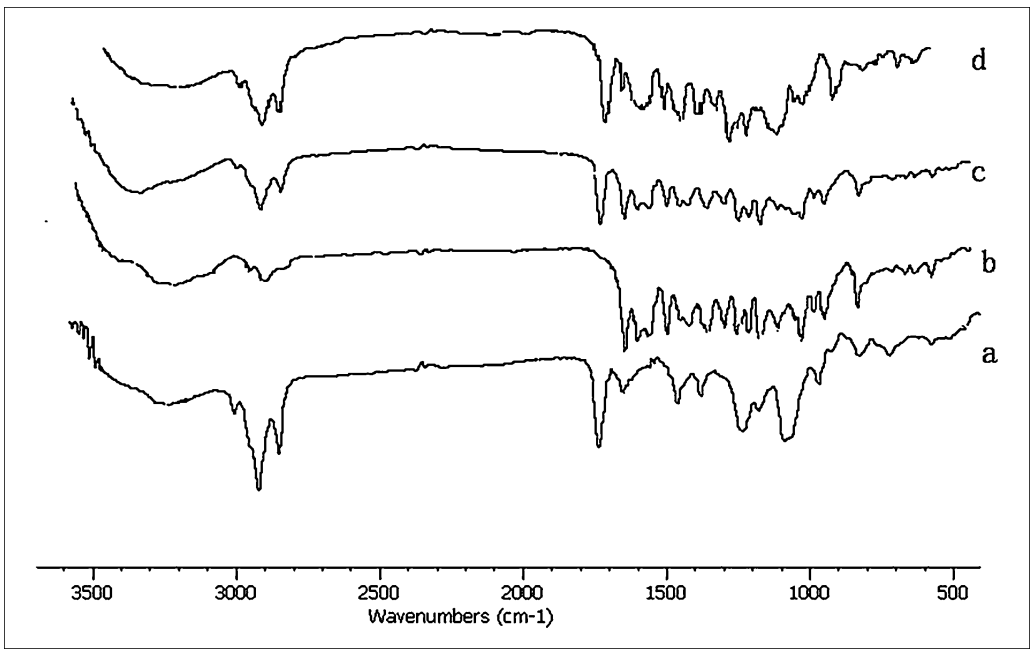

Fig. 3: IR spectra of phospholipid (a), icariside II (b), physical mixture (c) and icariside II–phospholipid complex (d)

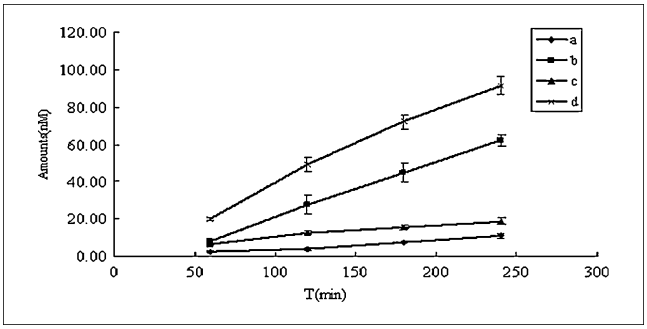

Fig. 4: The absorption of icariside II as time changes, c a icariside II A-B b icariside II B-A. c icariside II–phospholipid complex A-B d icariside II–phospholipid complex B-A Cumulative amounts of icariside II transported across Caco-2 cell monolayers. The cumulative amount transported at each time point using the Kruskal–Wallis test followed by Tamhane's post hoc was used to analyze the data statistically. (n = 3)

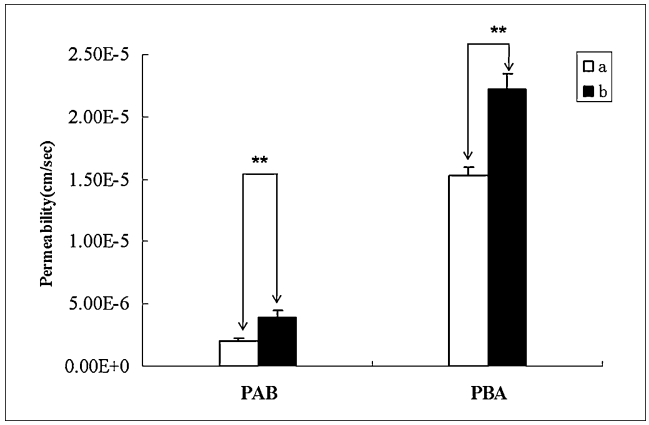

Fig. 5: Permeabilities of icariside II(a) and icariside II–phospholipid complex(b). The rates of transport were used to calculate permeabilities using Eq. (1) and the calculated permeabilities are plotted here as bars. Each bar represents the average of three determinations and the error bars are the standard deviation of the means.The asterisk symbol indicates a statistically significant difference between permeability icariside II and icariside II–phospholipid complex.The number of asterisk symbol indicates the level of significance with $^{**}p < 0.01$ and $^{*}p < 0.05$. One-way ANOVA with Tamhane's post hoc was used to analyze the data statistically

that drugs and phospholipids combined and formed some kind bonds, such as hydrogen bonds or van der Waals forces. The infrared spectra of the complex indicated that some characteristic absorption peaks are changed, and we can infer that it is icariside II–phospholipid complex instead of their physical mixture. By forming an icariside II–phospholipid complex, we can enhance the absorption of icariside II, which was linear with time, and would also increase secretion in Caco-2 cell monolayers. This was mainly due to the characteristics of phospholipids in the icariside II–phospholipid complex.

Not withstanding its limitations, this study does suggest the ratio of icariside II and phospholipids of (1:1) to prepare the complex and determine the absorption conditions. So it is still necessary to research absorption conditions of the complex at different ratios between icariside II and phospholipids. The bioavailability of icariside II–phospholipid complex also needs to be studied. Whether different kinds of phospholipids have different effects on absorption is also worthy to be investigated.

Despite its limitations, this study can clearly indicate a convenient method to prepare icariside II–phospholipid complex and make a new attempt to use Caco-2 cell monolayers to evaluate absorption of the icariside II–phospholipid complex. The complex could enhance the absorption of icariside II, as well as its excretion, which is first demonstrated in this article.

4. Experimental

4.1. Materials

Cloned Caco-2 TC7 cells were a kind gift from Dr. Moniqué Rousset of INSERM U178 (Villejuit, France). Icariside II (all purity > 98%) was provided by the Laboratory of Pharmaceutical Preparation (Jiangsu Provincial Academy of Chinese Medicine, China), and phospholipids were purchased from Tai-wei-yao-ye Ltd. Hanks' balanced salt solution (HBSS; powder form) were purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q water (Millipore, Bedford, MA) was used through out the study. Acetonitrile was of chromatographic grade (Merck Company Inc.UN).

4.2. Preparation of the icariside II–phospholipid complex

The complex was prepared by an anhydrous co-solvent reduction vaporization method. Briefly, icariside II powders and phospholipids were co-dissolved in anhydrous ethanol by gentle agitation until formation of a clear mixture. The resultant homogeneous solution was then rotary evaporated at a condenser temperature of 40 °C and under a vacuum of -0.1MPa to get the solid.

4.3. Determination of icariside II content in the complex

The content of icariside II in the complex was determined by a HPLC method as described. The content of icariside II in the phospholipid complex was determined as follows: 8.0 mg of phospholipid complex were dissolved in 10 ml of methanol, and a 20 μl aliquot of the resulting solution was injected into an HPLC system. The icariside II content in phospholipids complex (i.C.%) was calculated as follows:

i.C.% = (icariside II amount/phospholipids complex weight) × 100.

4.4. Phospholipid complex characterization

4.4.1. UV spectrum

Icariside II and icariside II–phospholipid complex were dissolved in methanol. In HPLC analysis**,** the DAD acquisition wavelength was set in the range of 200–400 nm.

4.4.2. Differential scanning calorimetry (DSC)

Samples were sealed in aluminum crimp cells and heated at a rate of 10 °C/min from 30 °C to 450 °C in a nitrogen atmosphere (DSC-60, SHI-MADZU, JAPAN). The peak transition maximum temperatures of phospholipids, icariside II, the mixture of phospholipids and icariside II and icariside II–phospholipid complex were compared using a Thermal Analyzer (TA-60WS, SHIMADZU, JAPAN).

4.4.3. IR spectra analysis

Using the KBr pellet method, taking desiccated phospholipid, icariside II, physical mixture, icariside II–phospholipid complex with KBr at the ratio of 1:100, respectively.

4.5. Cell culture

The Caco-2 TC7 cell line which is similar to wild-type Caco-2 cells was cultured in the laboratories of Dr. Moniqué Rousset of INSERM U178 (Villejuit, France), (Hu et al. 1999). However, it is more stable during the passage since it is a cloned cell line. The culture conditions for growing Caco-2 cells have been described previously (Chen et al. 2003; Hu et al. 2003; Hu et al. 1994). For the transport assay, cells were seeded on top of Transwell inserts in 6-well Transwell plates, which has a surface area of 4.2 cm^2, at a density of 100,000 cells/cm^2, growth media (Dulbecco's modified Eagle's medium supplemented with 10% fetalbovine serum), and quality control criteria were all basised on previously published reports (Chen et al. 2003; Hu et al. 2003, 1994). Culture medium was changed every 48 h. Cells were cultured for at least 21 days at 37 °C, 90% humidity and 5% CO_2, then used for transport studies between 21 and 23 day (Karamustafa 2006). Physiologically and morphologically well developed Caco-2 cell monolayers with transepithelial electrical resistance (TEER) values greater than 250 $\Omega \times cm^2$ were used for the experiments.

4.6. Transport experiments in the Caco-2 Cell culture model

Cell culture experiments were described previously. Briefly, after culture medium was aspirated, the cell monolayers were washed three times blank HBSS (pH 7.4). The transepithelial electrical resistance (TEER) values of cell monolayers were measured, which were more than 250 $\Omega \times cm^2$(Roger et al. 2009). The monolayers were incubated with the pH 7.4 blank HBSS for 1 h with 37 °C. Thereafter the incubation medium was aspirated. Afterwards, a solution containing the compound was loaded on to the apical or basolateral side. The amounts of transported compound were measured as a function

of time by UPLC method (methods to follow). Donor samples (400 μl) and receiver samples (400 μl) were taken at different times (typically 1 h) in triplicate, followed by the addition of 400 μl drug donor solution to the donor side or 400 μl of blank buffer to the receiver side. When comparing the permeability of icariside II and complex, each compound was used at the same concentration (10 μM) and the samples were taken at 0, 1, 2, 3 and 4 h after incubation. To each transport sample (400 μl), 100 μl of acetonitrile containing 3 μM of genistein was added as an internal standard and preservative, and then the resulting mixtures were vortexed for 30 s, centrifuged at 15,000 rpm for 15 min. The supernatant was analyzed by UPLC method (see below). At the end of the transport experiment, integrity of the monolayer was monitored by TEER value, and there is no significant change (Lev and Valery 2003).

4.7. Electrical resistance (cell integrity)

The integrity of the monolayers was determined by measuring the electrical resistance values at the end of each experiment. For this purpose, Bosstek type instrument with two electrodes was used and the results were expressed as $\Omega \times cm^2$ (Lo 2003).

4.8. Analytical methods

HPLC method: The stationary phase, a ZORBAX SB-C18 (4.6 × 250 mm, 5 μm) column, was used and kept at 30 °C. The mobile phase was a mixture of acetonitrile-water (75:25). The flow rate was 1.0 ml/min. Separarion was monitored at 270 nm.

UPLC method: UPLC method to detect the icariside II in the transport samples obtained from using the Caco-2 model. The conditions for UPLC analysis of icariside II of transport samples and icariside II–phospholipid complex samples were as follows: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm (Waters, Milford, MA, USA); mobile phase A, acetonitrile; mobile phase B, water contains 0.1%AcH; gradient, 0 to 0.9 min, 25% A, 0.9 to 1 min, 25% to 60% A,1 to 2 min, 60%A, 2 to 2.5 min, 60% A to 25%A, 2.5 to 3 min, 25%A; flow rate, 0.4 ml/min; colume temperature 35 °C; wavelength 270 nm; injection volume, 8 μl. The retention times for icariside II is 1.41 min. The retention times for genistein as an internal standard is 1.54 min.

4.9. Data analysis

Rate of transport is obtained from amount transported versus time curve using linear regression. The permeability of icariside II is calculated using the following equation:

$$P_{app} = \frac{V}{S \times C} \times \frac{dC}{dt} = \frac{1}{S \times C} \times \frac{dM}{dt} \quad (1)$$

Where V is the volume of the receiver (typical volume is 2.5 ml), S is the surface area of the cell monolayer (typical surface area is 4.2 cm^2), C is the initial concentration, $\frac{dC}{dt}$ is the rate of concentration change in the receiver side, and $\frac{dM}{dt}$ is the rate of drug transport. The rate of drug transport is obtained by linear regression analysis (a Microsoft Excel function).

4.10. Statistical analysis

All experiments were conducted at least in triplicate. Data are presented as mean ± S.D. The data were analyzed by Student's t-test. A one-tailed t-test (Microsoft Excel) ($P < 0.01$) was used to identify significant differences between permeability results with the control experiments.

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