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RESEARCH NOTE

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Comparison of the Permeability of Stilbene Analogues in Caco-2 Cells

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Abstract Permeability of resveratrol, piceid, rhapontigenin, and rhaponticin in Caco-2 cell assays using high-performance liquid chromatography were compared. Caco-2 cell monolayers were used to evaluate the transport rates of stilbene analogues from the apical to the basolateral sides. All stilbenes experimented in this study were transported to the basolateral side by times. For comparing the permeability of 4 stilbenes, we calculated the slope of the cumulative concentration of each stilbene in basolateral sides over time, resulting in those values of resveratrol, piceid, rhapontigenin, and rhaponticin with 3.766×10^{-5}, 4.330×10^{-6}, 5.430×10^{-5}, and 2.458×10^{-5} μM/sec, respectively. Apparent permeability coefficient of resveratrol and rhapontigenin were calculated to 9.994×10^{-6} and 1.441×10^{-6} cm/sec, respectively, while those of piceid and rhaponticin were to 1.149×10^{-7} and 6.523×10^{-7} cm/sec, respectively. These results suggest that aglycones would be absorbed more effectively than glycosides in stilbenoids.

Key words: Caco-2 cell, resveratrol, piceid, rhapontigenin, rhaponticin

Introduction

Stilbenes, non-flavonoid class of phenolic compounds, are naturally occurring phytochemicals that can be found in various foods, such as grape, wine, bilberry, cranberry, rhubarb, and peanut (1,2).

Resveratrol identified from grapes and wine has been the most widely studied and has been shown to be a potent anti-oxidant, anti-imflammatory, anti-cancer, and chemoprotective agent (3-10). Recently, piceid, glycosylated compound of resveratrol, was reported to inhibit the formation Aβ25-35 filbrils *in vitro* (11). Rhapontigenin and its glycosylated compound, rhaponticin, were abundantly found in Korean rhubarb rhizomes and have been used for preventing poor circulation, chronic inflammation, and allergies (12,13).

Recently, one of our greatest concerns is absorption, distribution, metabolism, and excretion of active components in human body, and especially, initial absorption is very important step for the effective pharmacological action of active components on its target. Caco-2 cell model is widely employed in absorption experiments in the pharmaceutical industry (14).

Caco-2 cell model is a standard screening tool for the prediction of intestinal drug absorption in humans and for mechanistic studies of drug transport (15), which is reliable, easy to carry out, and requires only small quantities of compounds (16). Caco-2 cells resemble small intestinal epithelial cells (17), and when grown to confluence, cell polarity and tight junctions are established and several active transport systems are expressed (16). P-glycoprotein, the product of the multi-drug resistance gene, and the multi-drug resistance associated proteins (MRP) are also expressed in the cell membrane of Caco-2 cells and induce a basolateral-to-apical flux of xenobiotic compounds (18,19). The apparent permeability coefficients measured for reference compounds across Caco-2 cell monolayers have shown good correlation with extent of *in vivo* absorption (20,21).

In this study, the permeability of resveratrol, piceid, rhapontigenin, and rhaponticin in Caco-2 cell assays using high-performance liquid chromatography (HPLC) were compared.

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Materials and Methods

Materials *Trans*-resveratrol and rhaponticin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Piceid was purchased from Sequoia (Sequoia Research Products Ltd., Pangbourne, UK). Rhapontigenin was obtained from Korea Research Institute of Chemical Technology (Daejeon, Korea). All organic solvents were purchased from Burdick & Jackson (SK Chemicals, Ulsan, Korea) and phosphoric acid was purchased from Sigma-Aldrich Co.

Caco-2 cell line HTB-37 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Trypsin-ethylenediamine tetraacetic acid (EDTA) (0.25%), Hanks' balanced salt solution, penicillin, and streptomycin were purchased from Welgene Co. (Daegu, Korea). Transwell polyester inserts (12 mm in diameter, 1.1 cm^2 surface area, and 0.4 μm pore size) were purchased from Corning Costar (Cambridge, MA, USA). Trans-endothelial electrical resistance (TEER) was measured using a millicell-electrical resistance system (ERS) instrument from Millipore (Bedford, MA, USA).

Cell culture Caco-2 cells were cultured at 37°C in Eagle's minimum essential medium with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 20 % fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity. Confluent monolayers were subcultured every 7 days by the treatment with 0.25% trypsin containing EDTA. The culture medium was 1.5 mL on the basolateral chamber and 0.5 mL on the apical chamber. To investigate the permeability of stilbene analogues in Caco-2 cell, cells were cultured for 21 days. The culture medium was changed every other day after seeding. The integrity of the monolayers of differentiated

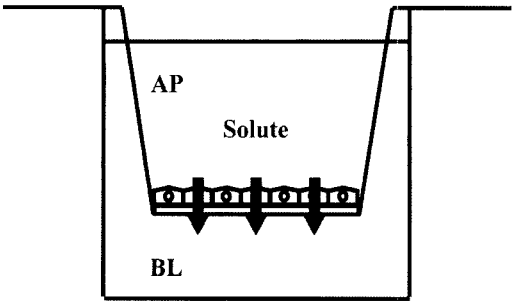
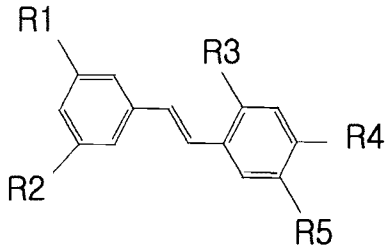


Fig. 1. Caco-2 cell assay model for intestinal absorption. A solution of a test compound is placed on the apical side (AP) of a Caco-2 cell monolayer, and the rates of appearance of the test compounds on the basolateral side (BL) of the cells are measured to assess the permeability of the monolayer for each compound.



Analogues	Mw	R1	R2	R3	R4	R5
Resveratrol	228	OH	OH	H	OH	H
Piceid	390	O-Glu	OH	H	OH	H
Rhapontigenin	258	OH	OH	H	OCH_3	OH
Rhaponticin	420	O-Glu	OH	H	OCH_3	OH

Fig. 2. Structures of resveratrol, piceid, rhapontigenin, and rhaponticin.

cells was monitored by measuring the TEER, and only monolayers with values >300 Ω/cm^2 were utilized.

Cell permeability and transport In preparation for the Caco-2 cell monolayer assays, the culture medium was removed from both the apical (AP) and basolateral (BL) chambers (Fig. 1). The cells were then washed 3 times and pre-incubated with Hanks' balanced salts (HBSS), pH 7.3, for 30 min at 37°C. A 10 mM stock solution of stilbene analogues in dimethyl sulfoxide (DMSO) was diluted to 50 μM in HBSS buffer (the final solutions of DMSO was less than 0.5%). These test solutions containing stilbene analogues were added to the apical chambers. The solutions in both chambers were removed at various time (5, 10, 20, 40, 60, and 80 min), and each stilbene was analyzed by HPLC and apparent permeability coefficient (*Papp*) of the compounds as transport rate were calculated using the below equation (14);

Apparent permeability coefficient (*Papp*)=$V_R \times dC/dt \times 1/ACo$

V_R: volume of recipient compartment (1.5 mL)
dC/dt: slope of the cumulative time
A: membrane surface area (1.1 cm^2)
Co: initial concentration in the donor chamber (50 μM)

HPLC analysis The analyses were performed on a Jasco HPLC system (Tokyo, Japan) comprising a quaternary pump (Jasco PU-2089 Plus), versatile autosampler (Jasco AS-2057 Plus), and column oven (Jasco CO-2065 Plus). The UV visible detector (Jasco UV-2075 Plus) was set at 308 nm. After injecting 20 μL of sample, separation was performed in a ZORBAX Eclipse XDB-C18 250×4.6 mm column (Agilent Technologies, Inc., Santa Clara, CA, USA).

The mobile phase for resveratrol and rhapontigenin used the combination of acetonitrile and water. The gradient elution filtered through a 0.45 mm Millipore filter (PVDF, Whatman, Clifton, NJ, USA) and degassed prior to use. The flow rate and column temperature were set at 0.6 mL/min and 25±1°C. The acetonitrile composition was initially set at 40% to 8 min, linearly decreased to 34% in 15 min and then increased to 40% in 20 min.

HPLC separation of piceid and rhaponticin was performed with the mobile phase consisting of solvent A, B, and C in gradient, where A was 1.5% phosphoric acid in water, B was 40% C in 1.5% phosphoric acid in water, and C was methanol, acetonitrile, and water (1 : 1 : 1, v/v/v). The gradient elution filtered through a 0.45 mm Millipore filter and degassed prior to use. Column compartment was set at 35±1°C. The flow rate was 1.0 mL/min. The linear gradient profile was from 50% solvent A in solvent B to 100% solvent B in 20 min and then 100% solvent C in 50 min, followed by washing and re-equilibrating column to initial condition. All experiments were performed at least 3 times and the results were expressed as mean±SD.

Results and Discussion

Resveratrol (Mw 228) has trihydroxyl groups in 3,5,4'-positions, and rhapontigenin (Mw 258) has trihydroxyl groups in 3,3'5-positions and methoxy group in 4'-position. Piceid (Mw 390) and rhaponticin (Mw 420) are glycosylated forms of resveratrol and rhapontigenin in 3-position, respectively (Fig. 2). Even though 4 stilbenes have similar structures, many researches elucidated different functional properties and proved to the potent pharmaceutical compounds (11,22). However, effective absorption in human intestine is most important step in pharmaceutical usage.

Caco-2 cell monolayers were used to evaluate the transport rates of stilbene analogues from the AP to the BL sides. All stilbenes experimented in this study showed a linear increase in BL by times, and especially, resveratrol was most rapidly transported in BL sides by time while piceid and rhaponticin, glycosylated forms, were transported slowly (Fig. 3). Li *et al.* (16) reported that resveratrol appeared to be rapid passive diffusion, and intestinal absorption following oral administration should pose no barrier to the bioavailability of resveratrol in humans. However, studies about the absorptions of other stilbenes were hardly found. In this study, we showed that the absorption tendency of rhapontigenin, rhaponticin, and piceid by time was similar with resveratrol in Caco-2 cell even though absorption rate was different.

For comparing the permeability of 4 stilbenes, apparent permeability coefficient of each stilbene was calculated. At first, we calculated the slope (dC/dt) of the cumulative

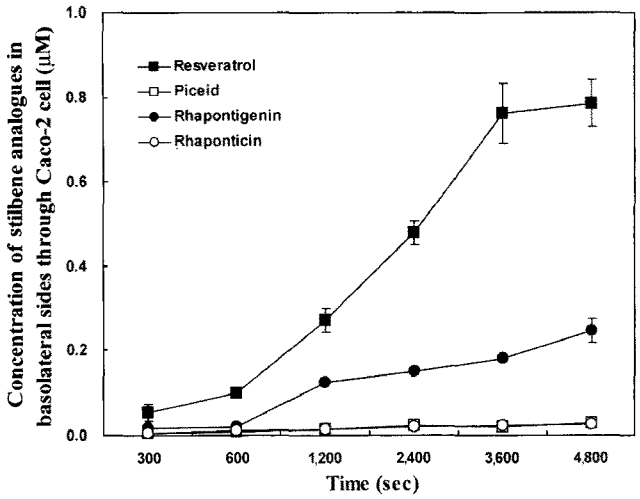


Fig. 3. Concentration of resveratrol, piceid, rhapontigenin, and rhaponticin in basolateral sides through Caco-2 cell.

Table 1. Apparent permeability coefficients of stilbene analogues through Caco-2 monolayers

Stilbenoids	dC/dt[1] (μM/sec)	P*app* (AP>BL)[2] (cm/sec)
Resveratrol	3.766×10^{-5}	9.994×10^{-6}
Piceid	4.330×10^{-6}	1.149×10^{-7}
Rhapontigenin	5.430×10^{-5}	1.441×10^{-6}
Rhaponticin	2.458×10^{-5}	6.523×10^{-7}

[1]Slope of the cumulative concentration of the compound in the recipient chamber over time.
[2]$Papp=V_R\times dC/dt\times/AC_0$; V_R, the volume of the recipient compartment (1.5 mL); A, the membrane surface area (1.1 cm^2); C_0, the compound initial concentration in the donor chamber (50 μM).

concentration of each stilbene in BL sides over time. dC/dt Values of resveratrol, piceid, rhapontigenin, and rhaponticin were 3.766×10^{-5}, 4.330×10^{-6}, 5.430×10^{-5}, and 2.458×10^{-5} μM/sec, respectively (Table 1). Using dC/dt values, P*app* of resveratrol and rhapontigenin were calculated to 9.994×10^{-6} and 1.441×10^{-6} cm/sec, respectively. Grès *et al.* (21) reported that P*app* $>2\times10^{-6}$ cm/sec in Caco-2 cell should be associated with efficient intestinal absorption. In this experiment, we proved that resveratrol and rhapontigenin were absorbed efficiently in human intestines. However, P*app* of piceid and rhaponticin was 1.149×10^{-7} and 6.523×10^{-7} cm/sec, respectively, which indicated that glycosylation of aglycone decreased the rate of permeability in human intestinal absorption. Many studies about bioavailability of flavonoids were reported (14,19). Walle *et al.* (19) reported that genistin is not transported across the Caco-2 model and, recently, Liu and Hu (14) reported that permeabilities of aglycones such as genistein were at least 5 times higher than their corresponding glycosides such as genistin in Caco-2 cell model, and our results showed that absorption of stilbenoids would be similar with the absorption of flavonoids.

In conclusion, we showed that aglycones such as resveratrol and rhapontigenin were rapidly absorbed rather than glycosides such as piceid and rhaponticin in Caco-2 cell model, and rhapontigenin as well as resveratrol would be effectively absorbed in human intestines. Recently, unknown stilbenoids were revealed and considered to the potent phytochemicals, and this study will provide the fundamental data for the pharmaceutical usage of stilbenoids in human body.

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