International Journal of Pharmaceutics 367 (2009) 58–64

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model

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ARTICLE INFO

Article history:
Received 17 June 2008
Received in revised form 8 September 2008
Accepted 15 September 2008
Available online 21 September 2008

Keywords:
Flavonoids
Caco-2 cell monolayer
Permeability
Structure–permeability relationship

ABSTRACT

To investigate the structure–permeability relationship of dietary/nutriceutic flavonoids, the transepithelial transport and cellular uptake of 36 flavonoids (including flavones, flavonols, dihydroflavones, dihydroflavonols, isoflavones, chalcones, flavanes, flavanols, methylated and glycosidic derivatives) were investigated using the Caco-2 cell monolayer. The apparent permeability coefficients (P_{app}) of the flavonoids were calculated from bilateral transport assays in the Transwell system with flavonoid determination using a high performance liquid chromatography (HPLC) coupled with a UV detector. The most flavonoids exhibited concentration-independent P_{app} values and a ratio of 0.5–1.8 for $P_{app\ AB\ to\ BL}/P_{app\ BL\ to\ AB}$, suggesting passive diffusion pathways. However, certain flavonoids e.g. morin and some flavonoid glycosides may involve the efflux mechanisms. For isoflavones, flavones, and dihydroflavones, the oil/water partition coefficients (additionally modified by the number and position of the three hydroxyl groups) was the key determinant for Caco-2 cell permeation. However, the permeability of flavonols is more complex with their structure possibly related to their high rate of cell accumulation. Overall, the parental skeleton structure, the number and position of free hydroxyl groups, accumulation and efflux in Caco-2 cell play the key roles in the transport of flavonoids across Caco-2 cell monolayer.

1. Introduction

Flavonoids are a large group of phenolic compounds that occur ubiquitously in foods of plant origin and considered as dietary supplements and functional ingredients of beverages, food grains, herbal remedies, and dairy products. According to the variations in the heterocyclic ring C, flavonoids are classified into flavones, flavonols, dihydroflavones, dihydroflavonols, isoflavones, chalcones, flavanes, flavanols, *etc.* The basic structure of flavonoids allows a multitude of substitution patterns in the benzene rings A and B within each class of flavonoids: phenolic hydroxyls, methoxyl, *O*- or *C*-glycosyl group(s), *etc.* To date, over 6467 different naturally occurring flavonoids have been described (Harborne and Williams, 2000), among which isoflavonoids has over 1000 different structures (Reynaud et al., 2005), and the list is still growing.

Flavonoids exhibit the wide spectrum of biological activities (Ren et al., 2003; Aron, 2008; Cook, 1996), including antibacterial, antiviral, antiallergic, anti-inflammatory and vasodilatory actions. Flavonoids were also shown to inhibit lipid peroxidation, cell fragility, and platelet aggregation, and modulate blood capillary permeability and the activity of cyclo-oxygenase and lipoxygenase. A considerable number of *in vitro* studies have sought to delineate structure–activity relationships (SAR) (Cao et al., 1977; Hammad and Abdalla, 1997; Arora et al., 1998; Heijnen et al., 2001) with hope to develop novel therapeutic/nutriceutic agents from flavonoid analogs. Since the biological efficacy depends greatly on oral bioavailability in the herbal drug/nutriceutic preparations, it is important to understand the molecular properties that limit oral bioavailability and to extrapolate SAR information.

The Caco-2 monolayer model has been well recognized for investigation of intestinal transport of xenobiotics (Hidalgo et al., 1989; Yamashita et al., 2000). Using the Caco-2 model, Kuo (1998) suggested that the transepithelial transport of flavones is a result of passive diffusion. Walle's group addressed a transporter-involving mechanism in the efflux of 4′-β-glucoside, (−)-epicatechin and (−)-epicatechin-3-gallate (Vaidyanathan and Walle, 2001,

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hank's Balanced Salts Solution; EBSS, Eagle's Balanced Salts Solution; NEAA, non-essential amino acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS, fetal calf serum; TEER, transepithelial electrical resistance; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; *p*-NPP, *p*-nitrophenylphosphate disodium salt hexahydrate; AP, apical; BL, basolateral; P_{app}, apparent permeability coefficient; O/W, oil/water; EC, (−)-epicatechin.

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doi:10.1016/j.ijpharm.2008.09.023

2003; Walgren et al., 2000a,b). Based on the studies on 11 flavonoids and alkyl gallates in Caco-2 cell monolayer model, Tammela et al. (2004) concluded that the degree of hydroxylation, molecular conformation, length of the side chain of flavonoids and alkyl gallates influenced membrane affinity as well as permeability; but the apparent permeability coefficients (P_{app}) for half of the flavonoids studied were not determined. Previously, we reported that Caco-2 cell monolayer exhibited efflux pathway for morin, isorhamnetin-3-*O*-rutinoside, and diosmetin-7-*O*-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside (Tian et al., 2006). Nevertheless, the mechanisms and the structure–permeability relationship for flavonoids were not well established.

In the present paper, we studied the structure–permeability relationship by determining the P_{app} values of 36 flavonoids with diverse structures as aglycones and their methylated and glycosidic derivatives (Fig. 1) including flavones, flavonols, dihydroflavones, dihydroflavonols, isoflavones, chalcones and (−)-epicatechin.

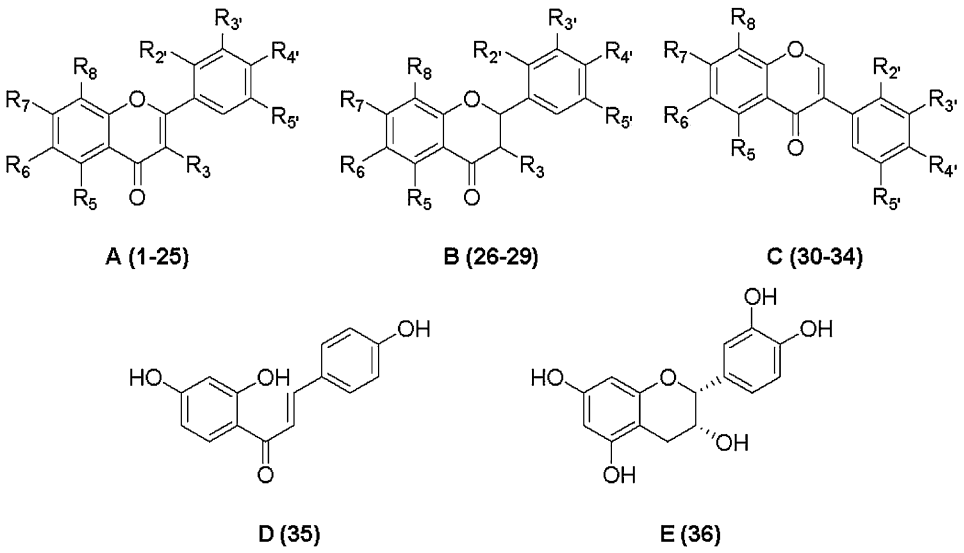

Fig. 1. The chemical structures of the tested compounds. A: flavones and flavonols type; B: dihydroflavones and dihydroflavonols type; C: isoflavones; D: isoliquiritigenin; E: epicatechin. Cglc: -*C*-glucopyranosyl; Oglc: -*O*-glucopyranosyl; glcA: -glucuronyl; DXG: diosmetin-7-*O*-β-D-xylopyra-nosyl-(1-6)-β-D-glucopyranosyl; XG: -7-*O*-β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl; DG: diosmetin-7-*O*-β-glucopyranoside; rha: -α-L-rhamnopyranosyl; IRR: isorhamnetin-3-*O*-rutinoside; RG: -α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranosyl.

	Compound	Type	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
1	Tangeretin	A	H	OMe	OMe	OMe	OMe	H	H	OMe	H
2	Wogonin	A	H	OH	H	OH	OMe	H	H	H	H
3	Chrysin	A	H	OH	H	OH	H	H	H	H	H
4	Acacetin	A	H	OH	H	OH	H	H	H	OMe	H
5	Apigenin	A	H	OH	H	OH	H	H	H	OH	H
6	Luteolin	A	H	OH	H	OH	H	H	OH	OH	H
7	Baicalein	A	H	OH	OH	OH	H	H	H	H	H
8	Scutellarein	A	H	OH	OH	OH	H	H	H	OH	H
9	DXG	A	H	OH	H	XG	H	H	OH	OMe	H
10	Isovitexin	A	H	OH	Cglc	OH	H	H	H	OH	H
11	Luteoloside	A	H	OH	H	Oglc	H	H	OH	OH	H
12	Baicalin	A	H	OH	OH	OglcA	H	H	H	H	H
13	DG	A	H	OH	H	Oglc	H	H	OH	OMe	H
14	Galangin	A	OH	OH	H	OH	H	H	H	H	H
15	Kaempferide	A	OH	OH	H	OH	H	H	H	OMe	H
16	Kaempferol	A	OH	OH	H	OH	H	H	H	OH	H
17	Tamarixetin	A	OH	OH	H	OH	H	H	OH	OMe	H
18	Quercetin	A	OH	OH	H	OH	H	H	OH	OH	H
19	Myricetin	A	OH	OH	H	OH	H	H	OH	OH	OH
20	Morin	A	OH	OH	H	OH	H	OH	H	OH	H
21	Quercitrin	A	Orha	OH	H	OH	H	H	OH	OH	H
22	Isoquercitrin	A	Oglc	OH	H	OH	H	H	OH	OH	H
23	IRR	A	ORG	OH	H	OH	H	H	OMe	OH	H
24	Myricitrin	A	Orha	OH	H	OH	H	H	OH	OH	OH
25	Rutin	A	ORG	OH	H	OH	H	H	OH	OH	H
26	Hesperetin	B	H	OH	H	OH	H	H	OH	OMe	H
27	Naringenin	B	H	OH	H	OH	H	H	H	OH	H
28	Eriodictyol	B	H	OH	H	OH	H	H	OH	OH	H
29	Taxifolin	B	OH	OH	H	OH	H	H	OH	OH	H
30	Formononetin	C	–	H	H	OH	H	H	H	OMe	H
31	Genistein	C	–	OH	H	OH	H	H	H	OH	H
32	Daidzein	C	–	H	H	OH	H	H	H	OH	H
33	Daidzin	C	–	H	H	Oglc	H	H	H	OH	H
34	Puerarin	C	–	H	H	OH	Cglc	H	H	OH	H

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), Hanks' Balanced Salts Solution (HBSS) and Eagle's Balanced Salts Solution (EBSS) were obtained from Gibco Laboratories (Life Technologies Inc., Grand Island, NY, USA). Non-essential amino acids (NEAA) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT, USA). Atenolol, propranolol, and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Alkaline phosphatase kit was purchased from Zhong Sheng Bei Kong Bio-Technology and Science Inc. (Beijing, China). *p*-Nitrophenylphosphate disodium salt hexahydratethe (*p*-NPP), penicillin and streptomycin were purchased from Amresco (Solon, OH, USA). The 36 flavonoids with IR, MS and NMR data were provided by the Sample Bank of Natural Products at the State Key Laboratory of Natural and Biomimetic Drugs, Peking University Health Science Center. The purity of flavonoids was >99% by high-performance liquid chromatographic (HPLC) assay. Other chemicals were of analytical grade. Transwell™ plates of 6 wells (24 mm, pore size 3.0 μm) and 12 wells (12 mm, pore size 3.0 μm) were purchased from Corning Costar (Cambridge, MA, USA).

2.2. HPLC assays of flavonoids

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprising a G1311A quatpump, a G1365B MWD UV detector coupled with an analytical workstation, a G1316A-colcom column oven and chiller, an on-line G1322A-degasser, an autosampler, and a 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) with a loop of 20 μL was used. The reversed-phase chromatography used an analytical Diamonsil™ ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm; Dikma, China) equipped with a C_{18} guard column (8 mm × 4 mm i.d., 5 μm; Dikma, China) cartridge system. The mobile phase consisted of A (MeOH)/B [0.15% potassium dihydrogen phosphate (KH_2PO_4) + 0.01 mM phosphoric acid (H_3PO_4)] (80/20–38/62) with 1.0 mL/min flow rate. Elution peaks were monitored with an UV detector at the wavelength of maximum absorption of each flavonoids at 200–400 nm. Peak area measurement was used to obtain standard calibration curves to determine each flavonoid.

Since the O/W partition coefficient (*P*) is proportional to the adjust retention time $t_{R'}$ in a reversed phase chromatography, $\log t_{R'}$ can be used as a representative for $\log P$. The $t_{R'}$ values were calculated as following from t_R obtained using one same column and the same HPLC conditions (A:B = 65:35, 1.0 mL/min) as described above:

$$t_{R'} = t_R - t_M = t_M \frac{P}{\beta}$$

where, t_M is the dead time and β is the column capacity.

2.3. Cell culture

Human colon adenocarcinoma cell line Caco-2 (ATCC #HTB-37) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Caco-2 cells were cultured in DMEM containing D-glucose (4.5 g/L), $NaHCO_3$ (3.7 g/L), supplemented with 10% FCS, 1% NEAA, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. All cells used in this study were between passages 50 and 62.

2.4. Permeation experiments

Caco-2 cells were seeded at a density of about 1×10^5 cells/cm^2 on a 6 wells Transwell insert filter and left to grow for 21 days to reach confluence and differentiation. The integrity and transportation ability of the Caco-2 cell monolayer were examined by measuring the transepithelial electrical resistance (TEER) with an epithelial voltohmmeter (EVOM, World Precision Instrument, Sarasota, FL, USA) and running standard assays using atenolol and propranolol as paracellular flux (Lennernas et al., 1996) and the transcellular flux marker (Artursson and Karlsson, 1991), respectively. Only cell monolayer with a TEER of above 500 Ω/cm^2 was used for the transport assays. Differentiation of Caco-2 cells was checked on the 3rd and 14th days by determining the activity of alkaline phosphatase with an assay kit (Lowry et al., 1951; Turowski et al., 1994; Rashid and Basson, 1996) and on the 21st day using transmission electron microscopy (Yang et al., 2007). These control assays were to confirm the Caco-2 cell monolayer was comparable to the small intestinal epithelium (Hidalgo et al., 1989; Artursson, 1990; Hilgers et al., 1990; Cogbrun et al., 1991).

After washing the Caco-2 cell monolayer twice with prewarmed HBSS medium (pH 7.4), the transport experiments were done by adding the flavonoid solutions to either the apical (AP, 0.5 mL) or basolateral side (BL, 1.5 mL) while the receiving chamber contained the corresponding volume of transport medium. After shaking at 55 rpm for 1 h at 37 °C in a water bath, samples were collected from both sides of Caco-2 cell monolayer and immediately frozen, lyophilized and preserved below −20 °C for subsequent HPLC analysis.

To determine the flavonoids, the lyophilized samples from both sides of the Transwell insert filter were dissolved in 200 μL MeOH, centrifuged at 130,00 × *g* for 10 min. Aliquot of 20 μL of the supernatant solution or cellular extracted flavonoids was used for assay by a reversed-phase HPLC as described above.

The flavonoid solutions were prepared by dilution of the stock solutions (10 mM in dimethylsulfoxide, DMSO) in EBSS (pH 6.0, for apical side loading) or HBSS (pH 7.4, for basolateral side loading) to 50 μM for vicinal-trihydroxyl flavonoids (**7**, **8**, **12**, **19** and **24**), or 15 μM for **4**, **15** and **17**, or 20 μM for **1**, **2** and **30**.

To measure the amount of cell accumulation including intracellular accumulation and passive binding to components of the cells, the cells were extracted after transport assays with 0.3 mL of MeOH. To stabilize the flavonoids, 20 μL of 0.1N phosphoric acid was added to the samples of vicinal-trihydroxyl flavonoids **7**, **8**, **12**, **19** and **24**.

The apparent permeability coefficients, P_{app} were calculated from the following equation,

$$P_{app} = \frac{\Delta Q/\Delta t}{AC_0}$$

where, $\Delta Q/\Delta t$ is the linear appearance rate of the compound on the receiver side (in mM/s), *A* is the membrane surface area (cm^2), and C_0 is the initial concentration in the donor compartment (in mM/cm^3).

2.5. Time- and concentration-dependent transport of the flavonoids across the Caco-2 cell monolayer

Five high permeable flavonoids (apigenin **5**; kaempferol **16**; naringenin **27**; genistein **31**; isoliquiritigenin **35**) were chosen to study the time- and concentration-dependence of Caco-2 cell monolayer permeability as representative of flavones, flavonols, dihydroflavones, isoflavones and chalcones. To observe the time-dependence of the selected flavonoids, 100 μM of flavonoid was added to either AP or BL side of the inserts. While shaking the sam-

Table 1
Apparent permeability coefficients (P_{app}) flavonoid compounds in the Caco-2 model.

Number	Compound	$P_{app\ A\ to\ B} \times 10^{-6}$ (cm/s)	$P_{app\ B\ to\ A} \times 10^{-6}$ (cm/s)	$P_{app\ A\ to\ B}/P_{app\ B\ to\ A}$
1	Tangeretin	29.30 ± 0.05	18.60 ± 0.90	1.57
2	Wogonin	23.20 ± 0.40	17.70 ± 1.30	1.32
3	Chrysin	16.00 ± 1.20	9.18 ± 1.21	1.74
4	Acacetin	14.30 ± 1.50	7.65 ± 0.24	1.86
5	Apigenin	10.90 ± 0.90	9.20 ± 0.66	1.19
6	Luteolin	8.87 ± 0.63	8.07 ± 0.34	1.10
7	Baicalein	7.29 ± 0.70	8.90 ± 0.53	0.82
8	Scutellarein	6.24 ± 0.64	6.72 ± 0.34	0.92
9	DXG	0.69 ± 0.08	1.58 ± 0.12	0.44
10	Isovitexin	0.63 ± 0.26	1.03 ± 0.44	0.61
11	Luteoloside	0.43 ± 0.10	0.51 ± 0.06	0.83
12	Baicalin	0.17 ± 0.05	0.51 ± 0.02	0.33
13	DG	0.12 ± 0.07	0.51 ± 0.08	0.23
14	Galangin	7.72 ± 1.14	6.95 ± 0.56	1.11
15	Kaempferide	6.44 ± 0.19	5.69 ± 0.33	1.13
16	Kaempferol	10.20 ± 0.46	6.93 ± 0.10	1.47
17	Tamarixetin	9.94 ± 0.55	7.37 ± 0.37	1.34
18	Quercetin	3.91 ± 0.21	3.47 ± 0.33	1.13
19	Myricetin	1.70 ± 0.18	2.12 ± 0.14	0.80
20	Morin	0.62 ± 0.07	2.50 ± 0.03	0.24
21	Quercitrin	0.79 ± 0.07	0.91 ± 0.05	0.86
22	Isoquercitrin	0.67 ± 0.04	0.97 ± 0.14	0.69
23	IRR	0.29 ± 0.03	5.14 ± 0.42	0.06
24	Myricitrin	0	0	–
25	Rutin	0	0	–
26	Hesperetin	18.30 ± 1.50	15.70 ± 0.90	1.16
27	Naringenin	12.60 ± 1.40	14.60 ± 0.69	0.86
28	Eriodictyol	5.29 ± 0.48	8.99 ± 0.45	0.59
29	Taxifolin	0.31 ± 0.04	0.59 ± 0.01	0.53
30	Formononetin	29.60 ± 0.60	24.00 ± 0.80	1.23
31	Genistein	22.50 ± 0.70	21.60 ± 1.40	1.04
32	Daidzein	21.00 ± 1.20	22.70 ± 0.30	0.92
33	Daidzin	0.44 ± 0.11	1.25 ± 0.36	0.35
34	Puerarin	0.40 ± 0.23	0.26 ± 0.16	1.54
35	Isoliquiritigenin	14.60 ± 0.10	9.35 ± 0.43	1.56
36	(−)-Epicatechin	0.60 ± 0.05	0.71 ± 0.04	0.85

$P_{app\ A\ to\ B}$: Transport of the flavonoids from apical to basolateral; $P_{app\ B\ to\ A}$: from basolateral to apical; Ratio ($P_{app\ A\ to\ B}/P_{app\ B\ to\ A}$): the ratio of $P_{app\ A\ to\ B}$ to $P_{app\ B\ to\ A}$. Data are means ± S.D. (n = 3–6). P_{app} value of about 2.18×10^{-5} cm/s for propranolol and 2.77×10^{-7} cm/s for atenolol. The concentration of test compounds was at 15 μM for **4**, **15** and **17**; at 20 μM for **1**, **2** and **30**; and at 50 μM for others flavonoids. The incubation time was up to 90 min.

ples (37 °C, 55 rpm), 100 μL aliquots were taken from BL receiver side at intervals from 0 to 90 min.

To determine the membrane permeation rate, the selected flavonoids (**5**, **16**, **27**, **31** or **35**) were added to the AP side. After shaking at 55 rpm and 37 °C for 40 min in a shaking water bath, aliquots were collected as described above.

3. Results

3.1. Transport of the flavonoids

The bilateral P_{app} values for various flavonoids have been summarized in Table 1. Permeation of **24** and **25**, across the Caco-2 cell monolayer was not detected, suggesting the P_{app} values for **24** and **25** less than 5×10^{-7} cm/s, the limit of flavonoid detection.

The P_{app} for the AP to BL flux of most flavonoid aglycons tested was above 10^{-6} cm/s, except **20** [$(6.2 \pm 0.7) \times 10^{-7}$ cm/s], **29** [$(3.1 \pm 0.4) \times 10^{-7}$ cm/s] and **36** [$(6.0 \pm 0.5) \times 10^{-7}$ cm/s]. These three compounds had permeability comparable to atenolol (2.77×10^{-7} cm/s), the paracellular transport marker (Lennernas et al., 1996). The ratios of $P_{app\ AP\ to\ BL}$ to $P_{app\ BL\ to\ AP}$ were within the range of 0.5–1.8; while the bilateral permeability ratios for **7**, **8**, **19**, **20**, **27** and **28** were less than 1.0, suggesting greater permeability in the BL to AP direction. The P_{app} values of flavonoid glycoside were lower than 10^{-6} cm/s with larger $P_{app\ BL\ to\ AP}$ except **34**.

Table 2
Cell accumulation of the flavonoids in the transport experiment from apical to basolateral.

Number	CA[a] (%)	Number	CA[a] (%)	Number	CA[a] (%)	Number	CA[a] (%)
14	60.07 ± 5.93	**1**	17.48 ± 1.30	**32**	6.20 ± 0.53	**23**	3.34 ± 0.15
15	58.25 ± 2.14	**31**	16.73 ± 0.52	**29**	6.12 ± 0.22	**25**	3.27 ± 0.22
4	37.18 ± 0.16	**30**	16.23 ± 0.69	**36**	5.88 ± 0.31	**10**	3.25 ± 0.53
16	32.84 ± 1.76	**27**	14.89 ± 0.79	**19**	5.60 ± 0.25	**8**	3.05 ± 0.49
18	30.10 ± 0.91	**2**	12.36 ± 0.22	**21**	5.40 ± 0.50	**34**	3.03 ± 0.16
17	25.22 ± 0.43	**26**	10.78 ± 0.47	**33**	4.31 ± 0.63	**9**	2.93 ± 0.22
6	20.99 ± 0.98	**28**	8.99 ± 0.72	**20**	4.20 ± 0.63	**24**	2.81 ± 0.22
35	18.79 ± 0.29	**3**	8.13 ± 0.53	**11**	4.17 ± 0.20	**13**	0.27 ± 0.02
5	18.16 ± 0.86	**7**	6.79 ± 0.20	**22**	3.40 ± 0.46	**12**	0.22 ± 0.01

[a] CA (Cell accumulation): the percent of the flavonoids that accumulated in the cell monolayer after transport experiment, including intracellular accumulation and passive binding to components of the cells. Data are means ± S.D., n = 3–6. The incubation time was up to 90 min.

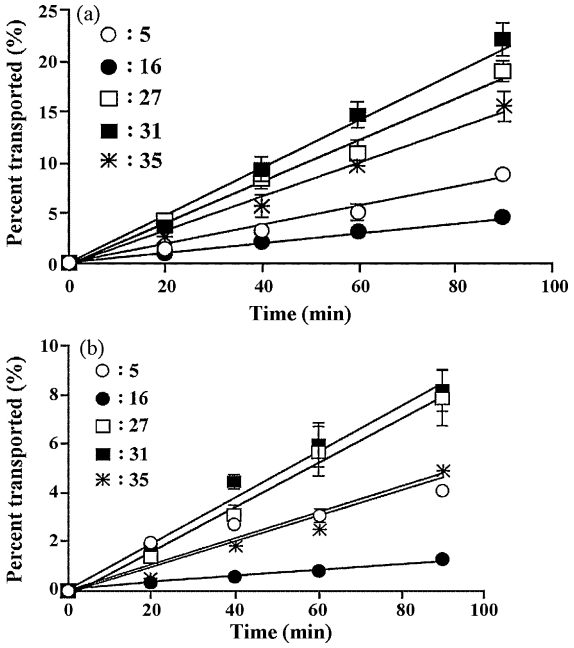

Fig. 2. The percent transported of the five flavonoid compounds in Caco-2cell monolayer as a function of time at 100 μM. (a) Apical to basolateral direction; (b) basolateral to apical direction. All experiments were carried out in triplicate. Data are the mean ± S.D. **5**: apigenin; **16**: kaempferol; **27**: naringenin; **31**: genistein; **35**: isoliquiritigenin

In summary Table 2 data, the isoflavone aglycons usually had higher P_{app} values than flavones, flavonols, dihydroflavones, dihydroflavonols, and chalcones. Methylated flavonoid aglycons had higher permeability than their corresponding flavonoid aglycon except **15**. Compound **1** exhibited exceptional high P_{app} of $(2.93 \pm 0.05) \times 10^{-5}$ cm/s.

3.2. The time course and concentration-dependence of membrane permeation

The bilateral permeation of the five selected flavonoids (apigenin **5**; kaempferol **16**; naringenin **27**; genistein **31**; isoliquiritigenin **35**) increased linearly with time (Fig. 2a and b) and the rates of membrane permeation increased linearly with the concentration within the test range of concentration (0–150 μM) while the P_{app} values basically remained unchanged with concentration.

3.3. Stability

The stability of flavonoid was expressed as the rate of reappearance, which is the percentage of the intact compound remained after incubation at 37 °C in HBSS for 1 h. It was found that **7**, **8**, **19** and **24** (having vicinal-trihydroxyl structure, stable in pH ≤2 solution) were unstable in pH 7.4 HBSS with the reappearance below 40%, because of auto-oxidation. The stability of these compounds increase with acidity of solution; the average reappearance became 60–80% in pH 6.0. The rest flavonoids tested were stable in pH 7.4 HBSS.

3.4. Cell accumulation and mass balance

Flavonoid glycosides exhibited very low rate (<5%) of cell accumulation. Isoflavonoids and flavonoids aglycone, except **4**, **6** exhibited an accumulation rate of <20%. For flavonol aglycon and methylated flavonol aglycon, the cell accumulation was, relative high (>25%). **14** and **15** exhibited the highest cell accumulation capacities of ~60% (Table 2).

To check the mass balance, the recovery of flavonoids during transport assays was measured as total amount of the flavonoids in both sides of the insert and in the Caco-2 cells. Isoflavone had a recovery rate of >95%. **1**, **2**, **4**, **14**, **15**, **26** and **27** showed recoveries between 80 and 95%. **3**, **5**, **6**, **16**, **17**, **20**, **28** and **35** exhibited recovery rates between 60 and 80%. **7**, **8** and **18** had recoveries between 55 and 60% while recovery of **19** was below 20%.

4. Discussion

The bilateral permeation across the Caco-2 monolayer of the selected five types of flavonoids (Fig. 1) increased linearly as a function of time up to 1.5 h (Fig. 2a and b). Therefore the estimate P_{app} value of the flavonoids in a 1 h assay described in Section 2 and the results have been listed in Table 1. The apparent permeability of compounds **7**, **8**, **19**, **24** could be underestimated due to poor stability of these compounds in transport buffers.

In Tables 1 and 2, most of the flavonoids exhibited a slightly larger AP to BL permeability (0.5–1.8 for the ratio of $P_{app\ AB\ to\ BL}/P_{app\ BL\ to\ AB}$) due to deviation of Transwell assay from the ideal sink system (Yang et al., 2004). However, compounds **12**, **13**, **20**, **23**, and **33** exhibited <0.5 of $P_{app\ AB\ to\ BL}/P_{app\ BL\ to\ AB}$ ratios, suggesting an efflux mechanism for these compounds. Among them, morin **20** has been shown to exhibit efflux in Caco-2 monolayer (Tian et al., 2006). Quercetin **18** has been indicated to undergo intestinal and/or hepatic glucuronidation and be pumped back by breast cancer resistance protein (BCRP) in the form of glucuronidated metabolites (Sesink et al., 2005; Zhang et al., 2007). In the present assays, efflux of quercetin was not observed probably due to the design of HPLC assays that only the parental compounds were determined. However, as quercetin is stable in solution, its significant mass imbalance (recovery <60%) implied metabolism and efflux for quercetin. Likewise, compounds **3**, **5**, **6**, **16**, **17**, **28** and **35** also could be potential substrates of either metabolic enzymes and/or efflux pumps for their low recoveries (60–80%); Previously, apigenin **5**, chrysin **3** and luteolin **6** have been indicated as substrates of UDP-dependent glucuronosyl transferase isoforms (Ng et al., 2004). Thus, metabolism and efflux transport by BCRP/MRPs are important for a variety of flavonoids especially the polar ones and further investigations would be appropriate.

It is observed that: (i) the rates of transportation of the five selected compounds (apigenin **5**; kaempferol **16**; naringenin **27**; genistein **31**; isoliquiritigenin **35**), which are lipophilic flavonoids, increased linearly with the concentration and saturation was not observed at the concentrations tested, indicating that the permeation process for these flavonoids was driven by the concentration gradient; (ii) the P_{app} values of the selected compounds remained basically the same with changes of flavonoid concentration; (iii) no efflux was observed except of the flavonoids **12**, **13**, **20**, **23**, and **33**. In addition, the magnitude of the bidirectional flux of glycosides was comparable to atenolol ($P_{app} = 2.7 \times 10^{-7}$ cm/s), suggesting paracellular transport for these glycosides (Lennernas et al., 1996). Since passive diffusion and carrier-mediated flux are known to be the two major pathways for a molecule to permeate across the intestinal epithelium (Stenberg et al., 2000), these results above indicate most of the flavonoids permeate across the Caco-2 monolayer via passive diffusion mechanisms.

Passive intestinal permeability depends on several factors, including lipophilicity, hydrogen bonding capacity, molecule size, *etc.* (Chan and Stewart, 1996; Ong et al., 1996). In general, the oil/water (O/W) partition coefficient, demonstrating the tendency for a molecule to partition into the lipophilic plasma membrane and

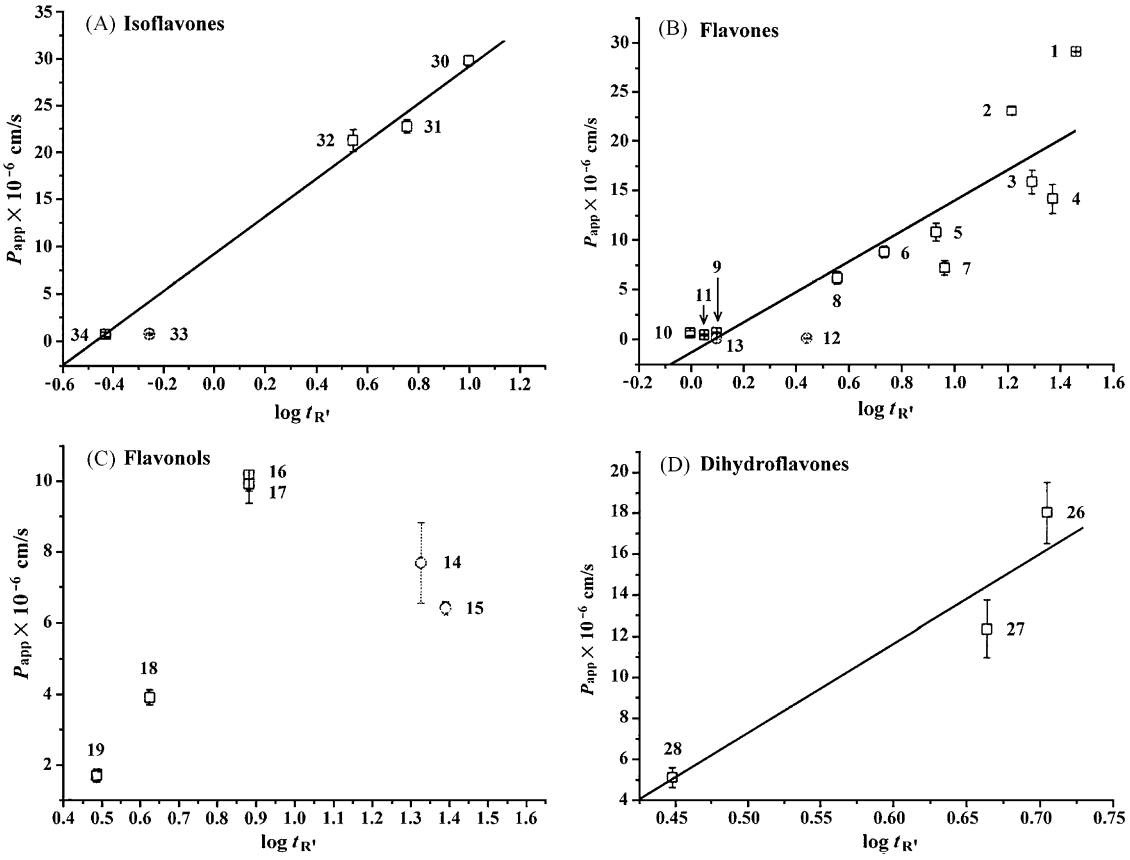

Fig. 3. Plot of P_{app} vs. log$t_{R'}$ for the types of isoflavones (A), flavones (B), flavonols (C), and dihydroflavones (D). The $t_{R'}$ is the adjust retention time for the flavonoids and log $t_{R'}$ can be a manifestation of the oil/water partition coefficient (log P).

also the capacity of forming hydrogen bond with water molecules, has been considered the major determinant. The partition coefficient (P) is difficult to obtain directly, but P is proportional to the adjust retention time $t_{R'}$ in a reversed phase chromatography. Therefore, the O/W partition coefficient (log P) could be reflected by log $t_{R'}$ of the compounds obtained under the same HPLC conditions using one same column. As seen in Fig. 3, isoflavones, flavones, and dihydroflavones exhibited linear correlations between P_{app} and $t_{R'}$, suggesting the O/W partition coefficient play the major role in membrane permeability for isoflavones, flavones, and dihydroflavones.

The P_{app} values increased adversely with the number of hydroxyl groups among a certain type flavonoids: the P_{app} values increased in an order of **6** < **5** < **3** among flavones; **19** < **18** < **16** (except of **14** and **20**) among flavonols. In addition, the position of the hydroxyl group also affected the P_{app} value: **16** had a larger P_{app} value compared with **6** although both had four hydroxyl groups but in different positions. These results agree with that the low bioavailability of dietary flavonoids is mainly due to highly efficient glucuronic acid and sulfate conjugation of these mono- or polyhydroxylated agents (Vaidyanathan and Walle, 2001, 2003; Walgren et al., 2000a,b; Tammela et al., 2004).

Methylated flavonoids with fewer hydroxyl groups exhibited larger P_{app} value compared with its aglycone (Table 1) with kaempferide **15** as an exception. Tangeretin **1** (having five methoxyl groups) showed a large P_{app} value, which is consistent with the previous postulation that flavone without hydroxyl group showed larger o/w partition coefficient and the higher P_{app} value (Tammela et al., 2004) and additionally, methylation on flavones could greatly increase the hepatic metabolic stability and intestinal absorption (Walle, 2007).

It is notable that the permeability of flavonols was not well correlated with their index for O/W partition coefficient—$t_{R'}$. These compounds normally exhibited a high rate of cell accumulation except **20** (efflux mechanism) and **19** (instable). Compounds **14** and **15** produced the highest cell accumulation among the flavonoids tested. The reason might be related to a 3-hydroxyl group at the C-ring in the structure. As proposed by Murota et al. (2002) and Dijk et al. (2000), those flavone aglycones with the planar structures normally showed higher membrane affinities. Nevertheless, the mechanisms for high cell accumulation needs to be investigated further.

Catechins are one group of the major flavonoids in grapes, wine, tea and cocoa products. Compared with (−)-epicatechin-3-gallate, (−)-epigallocatechin and (−)-epigallocatechin-3-gallate (Vaidyanathan and Walle, 2003), (−)-epicatechin (EC) are much stable. Probably due to large number hydroxyl groups in the structure, its permeability across the Caco-2 cell monolayer is poor ($P_{app} = 6.0 \times 10^{-7}$), which is in agreement with previous report (Vaidyanathan and Walle, 2001). One possible reason for the poor permeability might related to formation of catechin polymer due to the phenolic coupling reactions of epigallocatechin gallate (Bors et al., 2001).

5. Conclusion

While most of the flavonoids were transported across the Caco-2 cell monolayer by the passive diffusion, however, efflux

mechanisms may exist with a variety of flavonoids and flavonoid glycosides. For isoflavones, flavones, and dihydroflavones, the O/W partition coefficient (additionally modified by the number and position of the free hydroxyl groups) was the key determinant for Caco-2 cell permeation. However, relating the permeability flavonols to the cell accumulation is more complex with their structure and further investigation is suggested. In overall, the present results provided some useful information for establishing the permeability relationship for predicting the oral bioavailability of dietary/nutriceutic flavonoids.

Acknowledgements

This work was supported by National High Technology Research and Development Program of China (2002AA2Z343C, 2004AA2Z3783, 2006BAI06A 01-02) and Beijing Municipal Special-purpose Science Foundation of China (Z0004105040311). We thank Prof. John J. Hefferren of The University of Kansas for editing the manuscript.

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