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Prediction of intestinal absorption and metabolism of pharmacologically active flavones and flavanones

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Abstract—Three glycosilated flavonoids (diosmin, hesperidin and naringin) and respective aglycones were characterized in terms of their apparent ionisation constants and bidirectional permeability using the cellular model Caco-2 as well as the artificial membrane model PAMPA. Ionisation curves were established by capillary electrophoresis. It was confirmed that significant amounts of the aglycones are ionised at physiological pH whereas the glycosides are in the neutral form. Permeation was not detected for the glycosides in either the apical to basolateral or basolateral to apical directions confirming the need for metabolism before absorption through the intestinal membrane. The aglycones permeated in both directions with apparent permeabilities (P_{app}) in the range of 1–8×10^{-5} cm/s. The results from both in vitro methods correlated providing some evidence of passive transport however the hypothesis of active transport can not be excluded particularly in the case of diosmetin. Metabolism of the aglycones was detected with the cell model, more extensively when loading in the apical side. Some of the metabolites were identified as glucuronide conjugates by enzymatic hydrolysis.

1. Introduction

Flavonoids are a large group of polyphenolic compounds naturally occurring in several plants and fruits as glycosides or, less frequently, as their aglycones. They are abundant in human diet[1–4] and their role in the prevention of cancer and cardiovascular diseases has attracted substantial attention.[5–7]

Diosmin and hesperidin (Fig. 1) are respectively a flavone and a flavanone rutinoside that can be found mainly in citrus and, in the case of diosmin, in Hyssop and Rosemary.[8–11] The effects of these compounds on the improvement of muscular tone and vascular resistance to inflammatory processes, anti-oxidant activity and ability to quench oxygen free radicals involved in cancer are frequently mentioned.[8–18] In Europe, they are available under medical prescription for the treatment of illnesses such as chronic venous insufficiency[15,19] but despite the current claims of biological activity, there is no general recognition of these compounds as valuable medicines (in the USA, for example, they are commercialised only as food supplements).

Naringin (Fig. 1), a flavanone neohesperidoside mainly found in grapefruit, also exhibits different pharmacological properties such as anti-inflammatory, anti-oxidant, anti-microbial, anti-mutagenic, anti-carcinogenic, cholesterol lowering, and free radical scavenging.[20–23]

Solubility of these flavonoid diglycosides in aqueous solution is low, particularly in the case of diosmin, unless very high pH is used,[24] even in the presence of dissolution aids.

In addition, bioavailability is usually low[25] and permeation is thought to occur only after intestinal metabolism since the diglycosylated forms are not found in the systemic circulation. It is generally recognized that flavonoid diglycosides like rutinosides and neohesperidosides, pass intact through the small intestine.[26] In contrast to glucosides, which can be hydrolysed by glucosidases available throughout the intestinal tract, these diglycosides are hydrolyzed only by rahmnosidases

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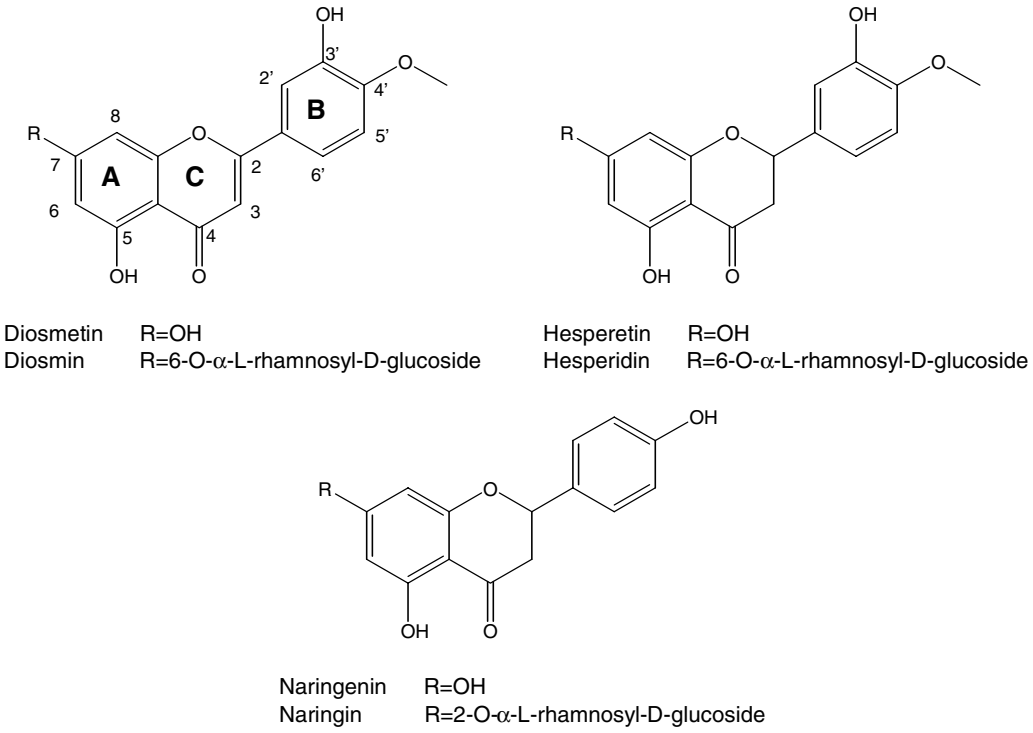

Figure 1. Structures of the flavonoids diosmin, hesperidin, naringin, and their aglycones.

produced by enterobacteria as they enter the cecum and colon. They are thought to be absorbed there as aglycones[27] which are found in the blood as conjugated metabolites such as glucuronides and sulfates.[28–31]

This led us to investigate pre- and post-deglycosilatyon membrane transport of these flavonoids using in vitro models. These models are becoming more popular for prediction of drug bioavailability[32,33] because they lend themselves to automation and high throughput screening, but also because they diminish the need to use animals in preliminary tests where many compounds are discarded. Moreover, being less complex systems they are easier to interpret and correlate with in vivo observations.

Among the in vitro systems used for the study of drug absorption, there are two models which find particular applicability: Caco-2[34] and PAMPA[35] (parallel artificial membrane permeability assay). In the Caco-2 model, permeability is tested across a differentiated monolayer of cells of the human colon adenocarcinoma, while in the PAMPA method an artificial phospholipidic membrane is used for the same purpose. Although cellular models provide more information in terms of active transport, efflux and metabolism, they are more time consuming than artificial membrane models such as PAMPA, which however provide only correlation to passive transport. Combining data from both models may nevertheless provide insights into the mechanism of absorption.

The main objective of the present study was the evaluation of the intestinal epithelial transport and the metabolism of diosmin, hesperidin, naringin and their respective aglycones, as a contribution to understanding the mechanisms responsible for the therapeutic effects of some flavonoid rutinosides.

Permeabilities were determined using the Caco-2 cell model and the artificial membrane model PAMPA. The observations were discussed taking into account other factors that can affect the rate of membrane penetration, like lipophilicity, degree of ionisation and molecular size.

The ionisation characteristics of the compounds were also determined since they have direct implications for membrane permeability. Despite being well known compounds, there is no published data relative to the ionisation constants (pK_a) of the test compounds except in the case of naringin and naringenin.[36,37] There are several methods for pK_a determination but due to its simplicity and selectivity, capillary electrophoresis (CE) has been frequently used[38–43] and was chosen for this purpose in the present work.

2. Results and discussion

2.1. Determination of the ionization profiles

The ionisation curves obtained by non-linear regression fitting of a sigmoidal curve to the effective mobilities of the test compounds against pH are shown in Figure 2. The aglycones possessed lower apparent ionisation constants than the corresponding glycosilated flavonoids (Table 1).

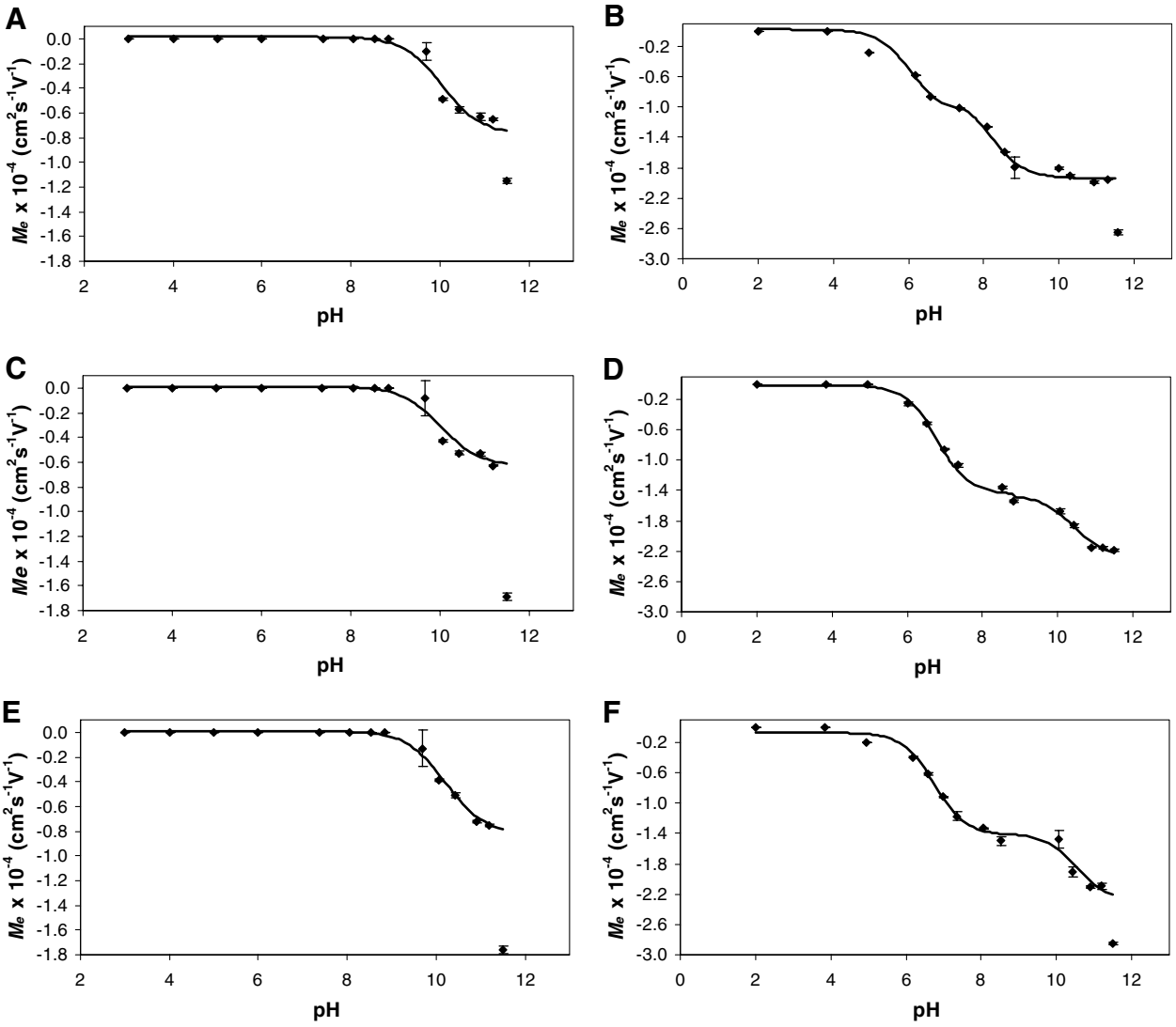

Figure 2. Ionisation curves ($n = 3$) obtained for the test compounds by capillary electrophoresis (A, diosmin; B, diosmetin; C, hesperidin; D, hesperetin; E, naringin; F, naringenin).

Table 1. Ionisation constants and absolute mobilities of mono (M_a^-) and double charged (M_a^{2-}) ions experimentally determined by capillary electrophoresis and estimated

	Diosmetin		Hesperetin		Naringenin	
	Experimental[a]	Estimated[b]	Experimental[a]	Estimated[b]	Experimental[a]	Estimated[b]
pK_{a1},	6.0 ± 0.1	7.29	6.8 ± 0.1	7.27	6.8 ± 0.1	7.27
M_a^-	−1.0E−4		−1.5E−4		−1.5E−4	
pK_{a2}	8.2 ± 0.2	9.54	10.4 ± 0.2	9.78	10.4 ± 0.2	9.47
M_a^{2-}	−1.9E−4		−2.2E−4		−2.3E−4	
pK_{a3}	>11.50	11.28	>11.50	11.31	>11.50	11.31
	Diosmin		Hesperidin		Naringin	
pK_{a1}	10.1 ± 0.2	9.39	10.0 ± 0.2	9.56	10.2 ± 0.1	9.34
pK_{a2}	10.1 ± 0.2	10.07	10.0 ± 0.2	10.17	10.2 ± 0.1	9.94
M_a^{2-}	−6.5E−5		−5.4E−5		−7.6E−5	
pK_{a3}–pK_{a8}	>11.50	⩾12.36	>11.50	⩾12.36	>11.50	⩾12.52

[a] Sigmoidal adjustment of CE mobilities against pH (GraphPad Prism 5).
[b] MarvinSketch (http://www.chemaxon.com/marvin).

For the flavonoids studied, considering the number of ionisable OH groups present in each molecule, three pK_a values for the aglycones were expected (corresponding to the three phenolic OHs) and eight for the glycosides (two phenolic and six alcoholic OHs), as estimated using MarvinSketch program (Table 1).

However, since dissociation of the alcoholic OHs occurs only in strongly basic conditions, which are outside of the experimental pH range of this methodology, it was not possible to determine them. The curves obtained from the experimental data (Fig. 2), for the glycosides, only show one inflexion point, (ca. pH 10.0) and a second one is suggested at higher pH values (>11.5). The first mobility differential probably results from a doubly charged ion since the experimental data does not fit perfectly to Eq. (2) in the case of hesperidin and diosmin. In fact, the experimental curves are steeper than the adjusted ones in which the slope is constricted to 1. This is indicative of a faster increase in the effective mobility against pH, that is not consistent with the formation of a single charged ion. These results were expected since the predicted difference between pK_{a1} and pK_{a2} is less than one unit. Naringin afforded a better curve fit but since the determined absolute mobility (M_a) is in the same order of magnitude of those obtained for hesperidin and diosmin it is likely that it corresponds to a double charged ion as well. The better fit for naringin curve was probably resulted from the difference between pK_{a1} and pK_{a2}. The second mobility increase at pH values >11.5, which cannot be adjusted due to insufficient experimental data at higher pH, must correspond to the ionisation of the OH groups of the carbohydrate.

For the aglycones diosmetin and naringenin, as expected, three pK_a values were detected but only two could be accurately determined since the third inflexion point occurred at high pH values (>11.5). For hesperitin only two mobility steps were detected in the experimental pH range but the M_a and slopes of the curves are consistent with a single deprotonation in each case.

In the aglycones, pK_{a1} should correspond to the ionization of the 7-OH group since the negative charge resulting from deprotonation of this group will be stabilized by conjugation.

The deprotonation of the hydroxyl groups in ring B (3′-OH or 4′-OH) should correspond to pK_{a2} (pK_{a1} in the case of the glycosilated flavonoids) since deprotonation of the 5-OH group must be the hardest to occur due to hydrogen bonding with the vicinal carbonyl group. Also, the introduction of a second negative charge in ring A, in the case of the aglycones, would make it more unstable. This is also the reason why deprotonation of 5-OH in the aglycones (which corresponds to pK_{a3}) occurs at higher pH than in the glycosilated flavonoids (pK_{a2}) where position 7 is not ionizable.

2.2. Transepithelial transport experiments

Based on Caco-2 cell viability the highest concentration showing reduced cytotoxic effect (under IC_{50}) for all compounds was chosen for the transport studies (15 μM).

The stabilities of the donor solutions in Hank's Balanced Salt Solution (HBSS) at this concentration were studied. Samples analysed by HPLC at different times after preparation revealed that, after two hours, over 80% of the original amount of each compound remained in solution, however, after 24 h, no diosmin and only about 50% diosmetin was still in solution (Table 2). The permeability assays of diosmin and diosmetin were therefore performed under super-saturation conditions, however since the permeability assays take only two hours, the amount of compound remaining in solution during this period permitted the evaluation of permeability.

No transport across the cell layer or metabolism was detected in either direction with the glycosilated flavonoids, neither in the presence nor in the absence of glucose. The purpose of the assay in a glucose-free medium was to evaluate the possibility, suggested by other authors,[44,45] that glucose transporters could be responsible for the transport of flavonoid glycosides but this was not confirmed for the compounds in this study.

The aglycones permeated across the membrane to the acceptor compartments in both the apical to basolateral (AP–BL) and the basolateral to apical (BL–AP) assays. Figure 3A and B present the time course accumulation at the acceptor compartments in each case.

Based on the comparison of the ionisation curves of the aglycones and of the glycosides, it would be expected that permeation of the aglycones would be lower at the pH of the transport medium (7.4), because they are more extensively ionised. However opposite results were obtained and this is probably due to the aglycones' smaller size and higher lipophilicity as demonstrated by the octanol/water partition coefficients (log P) estimated with the software KowWin (http://www.syrres.com/esc/est_kowdemo.htm) (Table 3).

P_{app} values (Table 3) were obtained for the bidirectional transport of the aglycones using Eq. (3). The apparent permeabilities of the aglycones in the serosal direction ($P_{app,ab}$) were above 3×10^{-5} cm/s. This value is in the same order of magnitude of the apparent permeability of caffeine[32] which demonstrates that the compounds are highly permeable.

The $P_{app,ab}$ obtained for naringin and naringenin are in agreement with the ones found in the literature.[46]

Apparent permeabilities in the mucosal direction ($P_{app,ba}$) were lower than $P_{app,ab}$ for diosmetin and hesperetin while for naringenin $P_{app,ba}$ was slightly larger.

Table 2. Compounds (%) remaining in HBSS solution (15 μM) at selected time points after sample preparation

Flavonoid	20 min	60 min	120 min	180 min	24 h
Diosmin	102.8	n.d.	88.6	78.4	0.0
Hesperidin	99.8	n.d.	87.9	86.3	84.9
Naringin	98.3	n.d.	92.2	90.4	88.5
Diosmetin	100.1	96.5	101.2	98.4	50.9
Hesperetin	94.5	99.9	91.1	90.8	80.0
Naringenin	102.0	99.4	100.4	91.2	79.9

n.d., not determined.

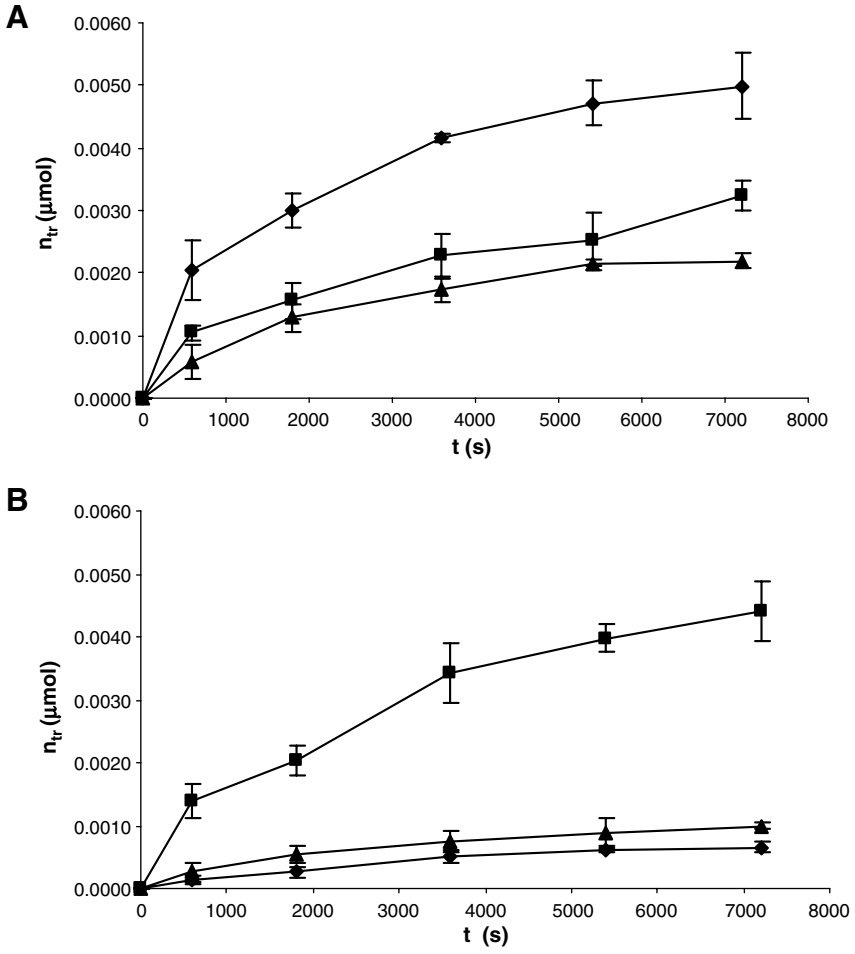

Figure 3. Cumulative amounts of the aglycones diosmetin (♦), hesperitin (▲) and naringenin (■) found in the receptor compartments in the apical-to-basolateral (A) and basolateral-to-apical (B) experiments. Error bars represent the confidence interval of three determinations.

Table 3. Apparent permeability values obtained for both AP–BL and BL–AP directions in the Caco-2 ($n = 3$) and in the PAMPA models ($n = 3$), molecular weights and estimated log P

Flavonoid	Caco-2 (AP–BL)		Caco-2 (BL–AP)		PAMPA		Log P[b]	MW
	P_{app} (10^{-6} cm/s)	Recovery (%)	P_{app} (10^{-6} cm/s)	Recovery (%)	P_{eff} (10^{-6} cm/s)	Recovery (%)		
Diosmin	[a]	61.9 ± 5.1	[a]	68.9 ± 3.1	[a]	n.d.	−0.72	608.6
Hesperidin	[a]	76.9 ± 7.4	[a]	80.2 ± 5.8	[a]	n.d.	−0.48	610.6
Naringin	[a]	85.7 ± 3.4	[a]	88.4 ± 7.2	[a]	n.d.	−0.52	580.5
Diosmetin	76.2 ± 5.6	59.1 ± 5.5	12.4 ± 2.3	66.8 ± 4.6	76.1 ± 3.5	82 ± 30	2.67	300.3
Hesperetin	47.1 ± 9.4	34.1 ± 1.3	27.0 ± 6.2	70.1 ± 6.1	35.7 ± 1.4	84 ± 12	2.44	302.3
Naringenin	37.8 ± 7.3	54.4 ± 2.1	51.4 ± 5.9	93.0 ± 7.6	25.7 ± 2.7	87 ± 14	2.61	272.3

n.d., not determined.
[a] Transport not detected.
[b] Predicted with KowWin software.

P_{ratio} values were calculated from Eq. (4) in order to investigate possible efflux or active transport. It is generally assumed that a P_{ratio} greater than 2 is predictive of relevant efflux, that is, the drug is actively pumped back into the intestinal lumen thus reducing its intestinal absorption.[47] The P_{ratio} values were 1.4, 0.6, and 0.2, respectively, for naringenin, hesperetin, and diosmetin. It is therefore unlikely that an efflux mechanism is involved in the permeation of these compounds and there is also no evidence of active transport in the case of naringenin and hesperitin. On the other hand, for diosmetin, the permeability rate was five times larger in the serosal direction and therefore the possibility of active transport should not be excluded.

The mass balances, expressed as recovery percentage (also presented in Table 3) and determined from Eq. (5), were higher in the BL–AP experiments. The incomplete recoveries may be justified by partial precipitation, in the case of diosmin and diosmetin, and by accumulation

of the compounds in the cells, as reported by other authors for similar compounds.[48] Lipophilic compounds, like the aglycones in this study, may be considerably retained by the cell monolayer.

2.3. Metabolism evaluation

Low recoveries can also be due to metabolism which was in fact observed for the aglycones, more extensively when loading at the apical side. These results, together with the fact that recoveries were higher in the case of the BL–AP assay, may indicate that metabolism occurs preferentially at the apical membrane.

Metabolites, D1, D2, and D3 for diosmetin, H1, H2, and H3 for hesperetin, and N1 for naringenin, were detected in both compartments but at higher concentration at the apical side in the AP–BL assays (Fig. 4). These metabolites are likely to be conjugated glucuronides and sulfates. Furthermore, the retention times of some of the metabolites produced by Caco-2 cells, matched the retention times of the glucuronides synthesised by enzymatic glucuronidation reaction using UGT supersomes.

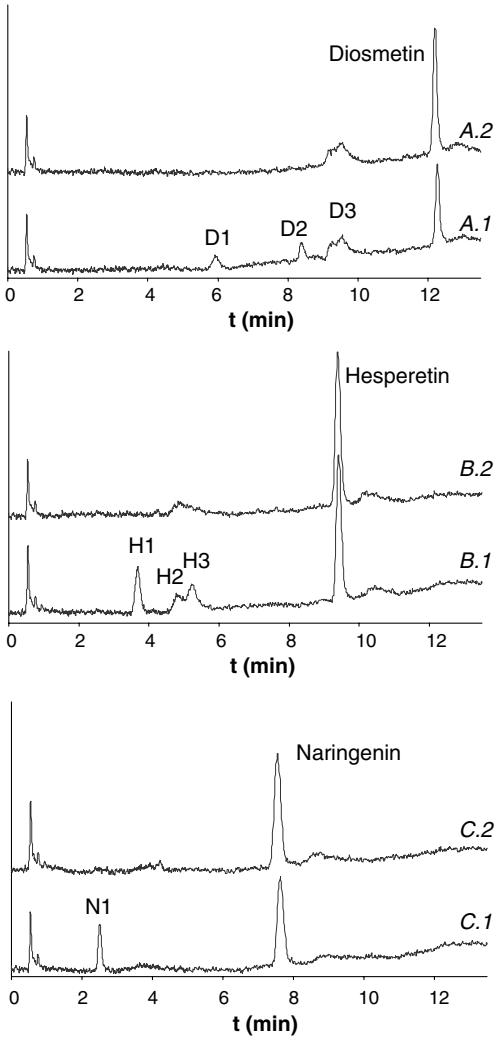

Figure 4. Metabolites of diosmetin, hesperetin and naringenin detected by HPLC in the apical solution after 120 min of apical loading (A.1, B.1, and C.1) and corresponding solutions after enzymatic hydrolysis (A.2, B.2, and C.2). Conditions as described in Experimental.

Confirmation was achieved by incubation of the samples obtained from the transport experiments with β-glucuronidase, which converted some of the metabolites back to the corresponding aglycones: D1, D2, H1, and N1 disappeared after incubation with β-glucuronidase from bovine liver while, at the same time, the peaks of the aglycones increased. H2 and D3 peaks were not affected.

2.4. Permeability studies through artificial membrane

In contrast with the aglycones, the glycosides were not transported across the soy lecithin lipidic membrane in the PAMPA model (Table 3). Soy lecithin was chosen as the phospholipidic component of the membrane because it has been referred to as affording the best correlations with human jejunal permeabilities.[49] In addition, the higher negative charge content of this type of membrane in comparison to other membranes such as egg lecithin can be an advantage for the transport of the compounds studied: at the pH used in these assays, the flavonoids are partially charged, and this may increase the repulsions between the sample and the negatively charged phospholipids, preventing H-bonding or other intermolecular forces, and consequently decreasing membrane retention.[49]

The effective permeabilities (P_{eff}) determined by this method predicted the highest rate of absorption for diosmetin, followed by hesperitin and then naringenin, which is in agreement with the data obtained using the Caco-2 model providing some evidence of passive transport. Membrane retention was less then 20% for all compounds.

Naringenin glucuronides obtained by enzymatic reaction were also tested in the PAMPA model but no transport was detected as expected due to their probable ionised state at the test pH.

3. Conclusions

To the best of our knowledge this is the first time that experimental ionization constants have been determined for diosmin, hesperidin and their aglycones. The pK_a obtained by CE in this study for naringin and naringenin are in good agreement with the ones found in the literature.[36,37]

Hesperidin, diosmin and naringin did not permeate, confirming other author's observations of low permeabilities for flavonoids glycosides and indicating that hydrolysis by intestinal microflora glycosidases may be necessary before absorption through the intestinal membrane.

The aglycones are more extensively ionized at physiological pH than the corresponding glycosides; however, the permeability results for the three aglycones studied,

that is, diosmetin, hesperetin and naringenin demonstrated that they are better absorbed through the intestinal epithelium. Absorption is most probably achieved passively for hesperitin and naringenin since bidirectional permeabilities were in the same order of magnitude, however in the case of diosmetin, the hypothesis of active transport should not be disregarded since P_{app} was significantly lower in the mucosal direction. On the other hand, the results obtained with the Caco-2 and the PAMPA models correlated well providing some evidence of passive transport. More experiments are needed in order to clarify this. The aglycones were moderately metabolized during the Caco-2 permeability experiments and therefore pre-systemic conjugation of the aglycones may be a relevant factor for limited bioavailability, unless metabolites are re-hydrolyzed.

4. Experimental

4.1. Test compounds

Diosmin (96.6%), hesperidin (94.5%), hesperetin (96.0%) were from Sigma–Aldrich (St. Louis, MO, USA). Diosmetin and naringenin (90%) were obtained from Extrasynthése (Genay, France). Naringin (95%) was from Fluka (Buchs, Switzerland).

Stock solutions of each flavonoid were prepared in DMSO (99.5%, Lab-Scan, Dublin, Ireland) at about 500 μM.

4.2. Determination of dissociation constants

Analyses were carried out in a Beckman P/ACE MDQ capillary electrophoresis system coupled with a diode array detector (DAD) (Palo Alto, CA, USA). A 75 μm, 50 cm (40 cm to detector) fused silica capillary was used and maintained at 25 °C.

The mobilities were determined using running buffers with an ionic strength of 0.05 and pH in the range of 2.0–11.5, prepared as described in Table 4. Sodium dihyrogenphosphate (99%, Merck, Darmstadt, Germany), sodium hydrogenphosphate (99%, Riedel-de Haën, Seelze, Germany), *ortho*-phosphoric acid (85%, Panreac, Castellar del Vallès, Spain), potassium hydrogenphosphate (99%, Sigma–Aldrich, Steinheim, Germany), potassium phosphate tribasic (⩾98%, Sigma–Aldrich, Steinheim, Germany), sodium acetate (99%, Riedel-de Haën, Seelze, Germany), acetic acid (99.79%, Fisher Scientific Ltd., Loughborough, UK), ammonia (25%, Panreac, Castellar del Vallès, Spain), ammonium chloride (>99.5%, Merck, Darmstadt, Germany), Trizma base (Sigma–Aldrich, Steinheim, Germany) and Trizma HCl (99%, Sigma–Aldrich, Steinheim, Germany) were used to prepare the buffer solutions. Sample solutions were prepared by dilution from stock solutions in water. Dimethyl sulfoxide (DMSO) was used as neutral marker (1%).

Table 4. Composition of running buffers used for the determination of the pK_a values by CE (I = 0.05 M)

pH range	Constituent	Stock solutions
2.5–3.0	Phosphate	0.5 M H_3PO_4, 1.0 M NaH_2PO_4
4.0–5.0	Acetate	1.0 M CH_3COONa, 1.0 M CH_3COOH
6.0–7.5	Phosphate	1.0 M NaH_2PO_4, 0.5 M Na_2HPO_4
8.0–9.0	Tris	0.2 M Tris, 0.2 M Tris.HCl
9.2–10.5	Ammonium	0.1 M NH_3, 0.1 M NH_4Cl
11.0–11.5	Phosphate	0.1 M K_3PO_4, 0.5 M K_2HPO_4

Prior to the assay of each compound, the capillary was rinsed with 0.1 mM NaOH, followed by water and the more basic running buffer. Mobilities were then determined in this buffer. Before proceeding to the determination of mobility with the buffer immediately following in the pH scale, the capillary was rinsed with it for 5 min. Injection was made by pressure (100 mBar) for 6 s and a potential of 20 kV was applied throughout the run.

The determination of ionisation constants by CE is based on the relationship between the electrophoretic mobility and the degree of dissociation of a species over a range of electrolyte pHs. The effective mobility, M_e ($cm^2\ s^{-1}\ V^{-1}$), of an ionic species at a particular pH, defined as the difference between the apparent mobility (M_{app}) and the mobility due to the electroosmotic flow (M_{EOF}), can be calculated using the following equation:

$$M_e = M_{app} - M_{EOF} = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (1)$$

where L_c is the length of the capillary to the detector (cm), L_d is the total capillary length (cm), V is the applied voltage (V) and t_{app} and t_{EOF} are the migration times (s) of the analyte and a neutral marker compound. For dilute solutions, a plot of effective mobility of an ionic species against the pH of the running buffer affords a sigmoidal curve (2) whose inflection point corresponds to the analyte pK_a.[40]

$$M_e = \frac{M_a - M_o}{1 + 10^{(pH - pK_a)}} + M_o \quad (2)$$

M_a represents the absolute mobility and M_o represents the mobility of the unionized species which is zero.

Each sample was tested three consecutive times at each pH and the mobilities were calculated based on the migration time of the sample and of the t_{EOF}, using Eq. (1).

The average of the mobilities of each sample was fitted by non-linear regression to a sigmoidal curve (2) using GraphPad Prism software (San Diego, CA 92130, USA).

Since the test compounds are multiprotic and the micro ionisation constants of the different ionisable groups in each molecule are quite close, it was only possible to determine the apparent ionisation constants. Whenever more than one inflexion point was distinctively determinable, the non-linear model used was run independently on different sets of data containing each inflexion point of the curves.

The micro pK_as of the test compounds were also estimated by molecular simulation using the software MarvinSketch.

4.3. Transepithelial transport experiments

Caco-2 cells from the German Collection of Microorganisms and Cell Cultures (DSMZ) were grown in RPMI-1640 media supplemented with L-glutamine and 10% fetal bovine serum (FBS), all from Gibco Invitrogen (Gran Island, NY, USA), at 37 °C in culture flasks (Nunc, Roskilde, Denmark) in a humidified atmosphere with 5% CO_2.

Before conducting the transport studies, the cytotoxicity of each test compound was examined at different concentrations using the CellTiter 96® AQueous Assay from Promega (Madison, WI, USA), which is composed of solutions of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine methosulfate (PMS).

This method is based on the capacity of viable cells to convert a tetrazolium salt (MTS) into aqueous soluble formazan in the presence of phenazine methosulfonate (PMS) which acts as an electron coupling agent. The quantity of formazan product, proportional to the number of living cells in culture, is measured by absorbance at 490 nm.

Cells were seeded in a 96-well plate at a concentration of 1.0×10^4 cells/cm^2 and incubated until they reached confluence. Then they were incubated for 2 h with flavonoid solutions in concentrations ranging from 0.5 to 100 μM prepared in culture medium, diluting from the stock solutions. The combined solution of MTS/PMS was prepared as described in the technical bulletin provided with the assay solutions. Then 40 μL were added to each well of the assay plate containing 200 μL of culture medium. After 2 h of incubation the absorbance at 490 nm was measured using a microplate spectrophotometer (Molecular Devices, SpectraMax Plus^{384}).

For transport studies, cells were grown in Transwell inserts (polycarbonate membrane, 12 mm diameter, 0.4 μm pore size, Corning Costar, NY) at a density of 1.0×10^5 cells/cm^2. The inserts were housed in 12-well plates containing culture buffer. Cells were used at passage 23–28, 21–23 days after seeding.

The integrity of Caco-2 monolayers was monitored routinely over the growing period, by measuring the Transepithelial Electrical Resistance (TEER) with an EVOM Epithelial Tissue Voltammeter (World Precision Instruments, USA). Inserts were used for transport experiments only when the values of TEER exceeded 400 Ω cm^2. TEER was also monitored after the transport experiments to confirm membrane integrity and it was over 400 Ω cm^2 in all cases.

The transport studies were conducted at 37 °C, replacing the culture medium by the transport medium (HBSS) in the presence or absence of glucose, consisting of 1.3 mM $CaCl_2$ (95%, Absolve), 5.4 mM KCl (99.5%, Riedel-de Haën, Seelze, Germany), 0.44 mM KH_2PO_4 (⩾95%, Riedel-de Haën, Seelze, Germany), 0.49 mM $MgCl_2$ (⩾98% Merck, Darmstadt, Germany), 0.41 mM $MgSO_4$ (99.5%, May & Baker, Dagenhan, England), 137 mM NaCl (⩾99.5%, Fluka, Buchs, Switzerland), 0.34 mM Na_2HPO_4 (⩾99%, Riedel-de Haën, Seelze, Germany), 5.5 mM D-glucose (⩾99%, Fluka, Buchs, Switzerland), and 4.2 mM $NaHCO_3$ (99.95 %, Fisher Scientific Ltd., Loughborough, UK) (pH 7.4).

Experiments were performed in triplicate in the AP–BL and BL–AP directions. The cell layers were washed twice for 30 min with warm HBSS before every experiment and then 15 μM solutions of each flavonoid, prepared in the transport media by dilution from the stock solutions, were added either to the apical or to the basolateral compartment. Samples were taken from the acceptor compartment, at selected times, ranging from 10 to 120 min, replacing always with an equal volume of HBSS. The solution at the donor compartment was also collected at the end of the experiment. Since it has been previously suggested[44,45] that glucose transporters could be responsible for the transport of flavonoid glycosides, an AP–BL assay in free-glucose medium was also conducted.

The P_{app} of each compound, expressed in cm/s, was determined according to the following equation:

$$P_{app} = J/A_S C_0 \quad (3)$$

where J is the rate of appearance of the compound on the acceptor compartment (μmole/s), C_0 is the initial concentration on the apical side (mM), and A_S is the surface area of the monolayer (cm^2).[50]

The permeability ratios (P_{ratio}) were calculated according to[47]:

$$P_{ratio} = P_{app,\,ba}/P_{app,\,ab} \quad (4)$$

The mass balance, expressed as the recovery percentage (R%), was determined according to the equation:

$$R\% = 100 \cdot (C_{a,\,120\ min} V_a + C_{d,\,120\ min} V_d)/(C_{d,\,0\ min} V_d) \quad (5)$$

where $C_{a,\,120\ min}$ and $C_{d,\,120\ min}$ are the concentrations measured at the end of the assay (120 min) in the acceptor and donor compartment, respectively; $C_{d,\,0\ min}$ is the concentration in the donor compartment at t_0; V_a and V_d are the volumes of the acceptor and donor compartments, respectively.[51]

4.4. Permeability studies through artificial membrane

The permeation studies through artificial membrane were done using the PAMPA model. This model consists of a 96-well microtitre plate (*p*ION, Woburn, MA, USA), which serves as the donor chamber, and a 96-well filter plate (MultiScreen-IP, Millipore), that serves as the acceptor compartment. The acceptor plate, placed directly on the donor plate, is bottomed by a hydrophobic microfilter disc (Immobilion-P membrane,

0.45 μm), impregnated with a phospholipid solution. A non-polar solvent such as *n*-dodecane is often used to dissolve phospholipids before applying on the filter.[52] In these tests, 5 μL of a 20% (w/v) solution of soy lecithin (Centrolex-R, Central Soya) in dodecane (98%, Fluka, Buchs, Switzerland) were used as phospholipidic membrane. Assays for each of the flavonoids were performed in triplicate by the double-sink method previously described.[49] Briefly, the method consists in two sets of experiments, one using a pH gradient between the acceptor and the donor reservoirs and the other using the same pH in both compartments. In the gradient experiment, a surfactant is added to the acceptor solution to create sink conditions while in the iso-pH experiment, a surfactant is added to the donor solution.

Acceptor and donor buffers were prepared with Na_2HPO_4 and NaH_2PO_4, to obtain a final phosphate concentration of 10 mM and the ionic strength was set at 154 mM by adding NaCl. For the iso-pH assay, solutions were at pH 7.4 in both compartments. The donor solution also contained the test compounds and 35 mM sodium dodecyl sulfate (SDS). For the pH gradient assays the buffer at pH 7.4 containing 35 mM SDS was used as acceptor solution while the donor solutions consisted of the test compounds in pH 6.8 buffer. In both assays, the concentrations of the flavonoids were 15 μM, prepared from dilution of the stock solutions and all solutions contained 10% DMSO. The model was placed in a minishaker (IKA) with a microtitre plate support which was set to 400 rpm. After 24 h the two plates were separated, the acceptor and the donor solutions were analyzed by HPLC and effective permeability was calculated according to the iterative method described by Avdeef.[49]

Permeation of naringenin glucuronide was also tested.

4.5. Identification of metabolites

For enzymatic synthesis of aglyones' glucuronides, Human UGT1A3 BD Supersomes Enzyme supplied with the corresponding UGT reaction Mixtures from BD Biosciences (San Jose, CA USA) was used as described in the technical bulletin provided with the assay solutions. In brief, a 0.2 mL reaction mixture containing 1 mg/mL protein, 2 mM uridine diphospho-glucuronic acid (UDPGA), 10 mM magnesium chloride, 0.025 mg/mL alamethicin, 50 mM Tris–HCl (pH 7.4) and 0.3 mM of the substrate was incubated at 37 °C. For naringenin and hesperetin the incubation time was around 7 h and for diosmetin 27 h. After incubation, the reaction was stopped by the addition of 94% acetonitrile/6% glacial acetic acid and centrifuged (10,000*g*) for 3 min. The supernatant was analyzed by HPLC.

Samples collected from the apical side of Caco-2 inserts at the end of the transport experiments were used for the identification of metabolites. For testing the presence of glucuronide metabolites, the pH was adjusted to 4.5 with 100 μL of sodium acetate buffer 1 M. β-glucuronidase from bovine liver (Sigma–ldrich, Steinheim, Germany) was added (1500 U) and the samples were incubated at 37 °C.[28] Control samples were incubated in the absence of enzyme. After 24 h of incubation, the samples were analysed by HPLC.

4.6. Quantification by HPLC

Aliquots obtained from the different studies were analyzed by HPLC and concentrations determined by the external standard method. The HPLC system used was a Merck-Hitachi, consisting of a L-6200 intelligent pump, a L-4200 UV–vis detector, an AS-2000A autosampler and a L-5025 oven. The injection volumes were 30 μL and the detector wavelength was set at 275 nm. Separation was performed on a Chromolith RP-18e analytical column (4.6 mm × 100 mm) at 3.0 mL/min and 35 °C. Eluent A was composed of acetonitrile (HPLC grade, Lab-Scan, Dublin, Ireland)—acetic acid (99.7%, Panreac, Castelar del Vallès, Spain)—methanol (HPLC grade, Lab-Scan, Dublin, Ireland)—water (Milli-Q grade) (1:4:18:74, v/v/v/v) and eluent B was methanol. Gradient elution started at 100% A for four minutes and then graded to 25% B in seven minutes, staying at the final composition for one minute. Solutions for calibration curves were prepared by further dilution of the stock solutions with DMSO/MeOH (1:1) to reach concentrations in the range 0.5–20.0 μM.

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