Methylated Flavonoids Have Greatly Improved Intestinal Absorption and Metabolic Stability

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ABSTRACT:

To better understand the relationship between the chemical structure and biological fate of dietary polyphenols, the hepatic metabolic stability and intestinal absorption of methylated polyphenols, in comparison with unmethylated polyphenols, were investigated in pooled human liver S9 fraction and human colon adenocarcinoma (Caco-2) cells. Consistent with previous in vivo studies, the two well known unmethylated polyphenols resveratrol (3,5,4′-trihydroxy stilbene) and quercetin (3,5,7,3′,4′-pentahydroxyflavone) were rapidly eliminated by the S9 fraction in the presence of the appropriate cofactors for conjugation and oxidation. In contrast, the methylated flavones, i.e., 7-methoxyflavone, 7,4′-dimethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4′-trimethoxyflavone, were relatively stable, indicating high resistance to hepatic metabolism. The corresponding unmethylated flavones, i.e., 7-hydroxyflavone, 7,4′-dihydroxyflavone, chrysos (5,7-dihydroxyflavone), and apigenin (5,7,4′-trihydroxyflavone), were rapidly eliminated because of extensive glucuronidation and/or sulfation just as resveratrol and quercetin were. The rate of intestinal absorption was evaluated using Caco-2 cells grown in porous inserts. The methylated flavones showed approximately 5- to 8-fold higher apparent permeability ($P_{\text{app}}$, 22.6–27.8 × 10⁻⁶ cm s⁻¹) of apical to basolateral flux than the unmethylated flavones ($P_{\text{app}}$, 3.0–7.8 × 10⁻⁶ cm s⁻¹). The lower $P_{\text{app}}$ values for the unmethylated flavones correlated with their extensive metabolism in the Caco-2 cells. Thus, combined use of the hepatic S9 fraction and Caco-2 cells will be useful for predicting the oral bioavailability of dietary polyphenols. The higher hepatic metabolic stability and intestinal absorption of the methylated polyphenols make them more favorable than the unmethylated polyphenols to be developed as potential cancer chemopreventive agents.

The potential utility of dietary polyphenols in chemoprevention of cancer, cardiovascular disease, and other diseases has eagerly been pursued (Middleton et al., 2000; Yang et al., 2001; Havsteen, 2002; Pervazi, 2003). Promising biological effects have been revealed in cell culture studies. However, when studies have been extended to the in vivo situation, in particular, in humans, using moderate, clinically relevant doses, this promise has not been fulfilled. This is clearly due to very low oral bioavailability, as has been shown in clinical studies of some of the more prominent polyphenols, for example, chrysos (5,7-dihydroxyflavone) (Walle et al., 2001), resveratrol (3,5,4′-trihydroxy stilbene) and quercetin (3,5,7,3′,4′-pentahydroxyflavone) (McAnlis et al., 1999; Williamson and Manach, 2005). Mechanistically, this can be related to the free hydroxyl groups of most polyphenols, giving rise to very rapid conjugation by glucuronidation and sulfation (Otake et al., 2002).

In a recent preliminary study, we found that two small dietary methylated flavones, 5,7-dimethoxyflavone (5,7-DMF) and 3′,4′-dimethoxyflavone (3′,4′-DMF), in sharp contrast to a typical unmethylated flavone, galangin (3,5,7-trihydroxyflavone), were metabolically stable in the S9 fraction of the human liver in the presence of the cofactors for glucuronidation and sulfation, as well as oxidation, suggesting that methylation protects dietary flavonoids from rapid hepatic metabolism (Wen and Walle, 2006b). This observation was confirmed in freshly plated human hepatocytes. Thus, this structural modification, occurring in certain plants, as well, provides a promising way to improve the bioavailability of dietary polyphenols.

In the present study, we extended the utility of the human hepatic S9 fraction as a model to characterize the metabolic stability of flavonoids to a number of methylated and corresponding unmethylated flavones (Fig. 1). We also examined the intestinal absorption of these flavones, using the human Caco-2 cell monolayer, a well accepted model for assessing the cellular permeability of potential drug candidates (Artursson and Borchard, 1997; Gan and Thakker, 1997; Walle et al., 2003). Taken together, this study clearly demonstrated the superior metabolic stability and transport of methylated versus unmethylated flavones, which should be critically important when selecting dietary flavonoids as potential chemopreventive agents in human disease.

ABBR EVIATIONS: 5,7-DMF, 5,7-dimethoxyflavone; 7-MF, 7-methoxyflavone; 7,4′-DMF, 7,4′-dimethoxyflavone; 5,7,4′-TMB, 5,7,4′-trimethoxyflavone; 7-HF, 7-hydroxyflavone; 7,4′-DHF, 7,4′-dihydroxyflavone; HBSS, Hanks’ balanced salt solution; HPLC, high-performance liquid chromatography; $P_{\text{app}}$, apparent permeability coefficient; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; $t_{1/2}$, elimination half-life; TFA, trifluoroacetic acid; UDPGA, uridine 5′-diphosphoglucuronic acid.
Materials and Methods

Materials. 7-Methoxyflavone (7-MF), 7-hydroxyflavone (7-HF), 7,4'-dimethoxyflavone (7,4'-DMF), 7,4'-dihydroxyflavone (7,4'-DHF), 5,7-dimethoxyflavone (5,7-DMF), and 5,7,4'-trimethoxyflavone (5,7,4'-TMF) (chemical purities 97–98%) were purchased from Indofine Chemical Co. (Somerville, NJ). Chrysin, apigenin (5,7,4'-trihydroxyflavone), quercetin and resveratrol (purities higher than 95%), and NADPH and uridine 5'-diphosphoglucuronic acid (UDPGA) were obtained from Sigma Chemical Co. (St. Louis, MO). Ultrapure (purity ≥99%) 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was obtained from S. S. Singer, University of Dayton (Dayton, OH). Fetal bovine serum was obtained from Atlas Biologicals (Fort Collins, CO). Pooled human S9 fractions from 15 donors were purchased from BD Gentest (Woburn, MA). D-[14C]Mannitol (0.1 Ci/l) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Other chemicals and reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

Metabolic Stability of Flavonoids in Pooled Human Liver S9 Fraction. For glucuronidation, the incubation mixture contained pooled human S9 fraction (50 μg of protein), 10 mM MgCl₂, 1 mM UDPGA, and 5 μM polyphenols (dissolved in methanol, final concentration 0.5%) in 100 μl of 50 mM Tris buffer (pH 7.4), as described previously (Otake et al., 2002; Wen and Walle, 2006b). After incubation at 37°C for 0 to 60 min, the reactions were terminated by adding 100 μl of cold methanol. Controls were incubated in the absence of UDPGA. To determine the sulfate conjugation of polyphenols, 5 μM polyphenols was incubated at 37°C for 0 to 60 min with pooled human S9 fraction (50 μg) in 100 μl of 50 mM Tris buffer (pH 7.4) containing 0.0625% bovine serum albumin, 8 mM dithiothreitol, and 0.1 mM PAPS (Otake et al., 2002). Controls were incubated in the absence of PAPS. The reactions were terminated by adding 100 μl of methanol, and the samples were centrifuged at 14,000g for 2 min. The supernatants (100 μl) were subjected to assay for the time-dependent polyphenol depletion using HPLC.

To determine the effects of a combination of glucuronidation, sulfation, and oxidation, the polyphenols were incubated with S9 fraction in the presence of three cofactors, i.e., 1 mM NADPH, 1 mM UDPGA, and 0.1 mM PAPS. Otherwise, the incubation conditions used were as described above, except for the addition of 10 mM MgCl₂ in the incubations.

Cell Culture. Caco-2 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma) and grown in a humidified atmosphere of 5% CO₂ at 37°C. After reaching 90% confluence, cells at passage 27-33 were used and seeded at a density of 1.0×10⁵ cells/cm². At 15 to 20 days after seeding, transepithelial electrical resistance values across the cell monolayers were measured using a Millicell-ERS voltohmmeter (Millipore Corporation, Billerica, MA). Insertions with transepithelial electrical resistance values ≥350 Ω cm² in culture medium were used for the experiments.

Transepithelial Permeability Experiments. The cells were washed twice for 30 min with warm Hanks’ balanced salt solution containing 25 mM HEPES, pH 7.4 (HBSS) (Walgren et al., 1998). The flavones (7-MF, 7-HF, 7,4'-DMF, 7,4'-DHF, 5,7,4'-TMF, and apigenin) were dissolved in ethanol and diluted to 5 μM with HBSS (final ethanol concentration 0.1%). For 5,7-DMF and chrysin, 10 μM was used to improve the sensitivity of HPLC. HBSS with 5 or 10 μM flavones (700 μl) was added to the apical chamber and 1800 μl of HBSS to the basolateral side. A small amount (50 μl) of [14C]mannitol (0.001 μCi/μl) was added to the apical chamber for assessment of the monolayer integrity. Samples (150 μl) were withdrawn from both chambers for analysis of flavones and their metabolites by HPLC after 1, 3, and 6 h of incubation. Aliquots of basolateral medium (500 μl) were analyzed for mannitol transport by liquid scintillation counting at the end of the experiment.
Previous studies have demonstrated that some flavonoids consumed in our diet may reach concentrations as high as 50 μM or more in the intestinal lumen (Walgren et al., 1998). Because of presystemic intestinal metabolism (Walle et al., 1999), the concentrations seen by the liver may only be a fraction thereof. Because plasma or tissue concentrations of flavones in general have not been measured in vivo, the use of 5 to 10 μM in this study is an educated guess of what may be encountered after consumption of various foods or spices.

**Metabolic Stability of Methylated and Unmethylated Polyphenols in Pooled Human Liver S9 Fractions.** Both the flavonoid quercetin and the phytoalexin resveratrol are unmethylated polyphenols, which have been shown to have very low oral bioavailability in humans due to extensive metabolism (McAnlis et al., 1999; Goldberg et al., 2003; Walle et al., 2004; Williamson and Manach, 2005). Therefore, these two compounds were used as standards to test their metabolic profiles using the S9 fraction. In good agreement with in vivo observations, both quercetin and resveratrol showed rapid disappearance in the presence of the cofactors for glucuronidation, sulfation, and oxidation, i.e., UDPGA, PAPS, and NADPH (Fig. 2, A and B). The depletion of quercetin was more rapid than that of resveratrol, with a t1/2 for quercetin of 5.3 min and for resveratrol, 10.2 min (Table 1).

We then determined the metabolic stability of four methylated and their corresponding unmethylated flavones with various chemical structures using the S9 fraction. All of the tested methylated flavones, i.e., 7-MF, 7,4'-DMF, 5,7,4'-DMF and 5,7,4'-TMF, showed much higher metabolic stability than their corresponding unmethylated anologs. The data are expressed as means ± S.E.M. from three or more determinations. Differences between methylated and the corresponding unmethylated flavones in P_app or flavone concentration at each time point were evaluated using Student’s t test. A p value <0.05 was considered to be statistically significant.

**HPLC Analysis.** All samples were analyzed by reverse-phase HPLC using a Millennium HPLC system (Waters Corp., Milford, MA) equipped with a photodiode array detector (model 996) and a Symmetry C18 column (3.9 × 150 mm; Waters). The flow rate was 0.9 ml/min. The mobile phase consisted of 60% methanol in 0.3% trifluoroacetic acid (TFA) with UV detection at 307 nm for 7-MF, 326 nm for 7,4'-DMF, 268 nm for 5,7,4'-DMF, and 331 nm for 5,7,4'-TMF, respectively. For 7-HF and apigenin, the mobile phase was 50% methanol in 0.3% TFA with UV detection at 312 and 338 nm, respectively. Chrysin was detected at 268 nm with mobile phase of 55% methanol in 0.3% TFA, 7,4'-TMF at 331 nm with 40% methanol in 0.3% TFA, and resveratrol at 306 nm with 32.5% methanol in 0.3% TFA. The mobile phase for quercetin and the phytoalexin resveratrol are unmethylated polyphenols, which have been shown to have very low oral bioavailability in humans due to extensive metabolism (McAnlis et al., 1999; Goldberg et al., 2003; Walle et al., 2004; Williamson and Manach, 2005). Therefore, these two compounds were used as standards to test their metabolic profiles using the S9 fraction. In good agreement with in vivo observations, both quercetin and resveratrol showed rapid disappearance in the presence of the cofactors for glucuronidation, sulfation, and oxidation, i.e., UDPGA, PAPS, and NADPH (Fig. 2, A and B). The depletion of quercetin was more rapid than that of resveratrol, with a t1/2 for quercetin of 5.3 min and for resveratrol, 10.2 min (Table 1).

We then determined the metabolic stability of four methylated and their corresponding unmethylated flavones with various chemical structures using the S9 fraction. All of the tested methylated flavones, i.e., 7-MF, 7,4'-DMF, 5,7,4'-TMF, showed much higher metabolic stability than their corresponding unmethylated anologs in the presence of the appropriate cofactors (Fig. 2, C–F, filled
symbols). Less than 20% of the parent compounds were eliminated after 60 min of incubation. In contrast, all unmethylated flavones were rapidly eliminated (Fig. 2, C–F, open symbols), with elimination 1/2 values of 4 to 10 min (Table 1); i.e., similar to quercetin and resveratrol. Among the unmethylated flavones, chrysin completely disappeared after 20 min, followed by 7-HF and apigenin. 7,4'-DHF was the most metabolically stable among these unmethylated flavones, but it still completely disappeared after 60 min of incubation; i.e., similar to resveratrol.

The relative importance of the individual metabolic pathways responsible for eliminating the flavones was assessed by measuring the disappearance of parent compound in the presence of either UDPGA or PAPS. Glucuronidation clearly predominated over sulfation for quercetin, chrysin, and apigenin, whereas sulfation predominated for resveratrol (Table 1). For 7-HF and 7,4'-DHF, there was an approximately equal contribution by sulfation and glucuronidation. These observations were confirmed by measuring the individual metabolites after incubation of the flavones with the individual cofactors (data not shown).

### Transport of Methylated and Unmethylated Flavones by Caco-2 Cells.

The transepithelial transport of methylated and corresponding unmethylated flavones was evaluated with Caco-2 cells grown in Transwell inserts on permeable membranes. After loading 5 or 10 μM flavones in the apical chamber, considerably higher concentrations of methylated flavones were observed in the basolateral chamber, in comparison with unmethylated flavones (Fig. 3, E–H, filled versus open symbols), indicating more effective intestinal absorption of the methylated compounds. The P_app values of apical to basolateral flux for methylated and unmethylated flavones, calculated from these observations, were 22.6 to 27.6 × 10⁻⁶ cm s⁻¹ and 2.2 to 6.1 × 10⁻⁶ cm s⁻¹, respectively (Table 2). These P_app values were much higher than that of the paracellular transport marker mannitol (approximately 0.4 × 10⁻⁶ cm s⁻¹), the latter validating the cell system integrity and indicating that methylated flavones have highly efficient absorption through the Caco-2 cell layer. It should also be noted that the disappearance of the methylated flavones from the apical chamber was slower (Fig. 3, A–D, filled symbols) compared with the unmethylated analogs (Fig. 3, A–D, open symbols), presumably reflecting the slower metabolism of the methylated flavones.

![Graphs showing transport of methylated and unmethylated flavones by Caco-2 cells.](image-url)
fact, there were no detectable metabolites of 5,7-DMF or 5,7,4'-HM1 or 7-MF and 7,4'-H11032-DMF, during the 6-h time course used.

Figure 4 shows the metabolites of the unmethylated flavones both in the apical (Fig. 4, A–D) and basolateral chambers (Fig. 4, E–H) after apical loading of the flavones. The concentrations of metabolites were much higher in the apical chamber than in the basolateral chamber, consistent with previous reports (Walle et al., 1999; Hu et al., 2003; Kaldas et al., 2003). Based on the data shown in the S9 fraction, the metabolites M1 and M2 were identified as glucuronic acid conjugates and M3 as sulfate conjugates. In the apical chamber, the concentration of the sulfate conjugates of all the unmethylated flavones in the apical chamber was much higher than that of the glucuronic acid conjugate(s), indicating different metabolic profiles in Caco-2 cells compared with the hepatic S9 fraction.

**Discussion**

This study demonstrates that methylation of dietary flavonoids, and probably other polyphenols, may result not only in a dramatic increase in their hepatic metabolic stability but also in great improvement of their intestinal absorption, both of which should greatly increase their oral bioavailability. In addition, this study shows the utility of the human hepatic S9 fraction and intestinal Caco-2 cell monolayer as effective models to establish these important properties.

As emphasized in many studies, the poor bioavailability of dietary polyphenols is highly dependent on their free hydroxyl groups, making them susceptible to glucuronidation, in particular, and sulfation, but not cytochrome P450 oxidation (Otake et al., 2002). Thus, blocking the free hydroxyl groups by methylation removes the influence of the highly efficient conjugation pathways, limiting the metabolic clearance in the intestinal epithelial cells as well as in the liver, as evidenced by the present study. In the presence of the cofactors for conjugation and oxidation, the elimination of the unmethylated polyphenols, i.e., six compounds studied here (Fig. 1) and one (galangin) in a preliminary study (Wen and Walle, 2006b), all displayed high stability in the human liver S9 fraction, with elimination half-lives of approximately 4 to 10 min (Table 1). There did not seem to be any difference in this respect between flavonoids with a single free hydroxyl group (7-HF) or five (quercetin). For most unmethylated flavonoids such as quercetin, chrysin, and apigenin, glucuronidation was the more efficient metabolic pathway; for resveratrol, it was sulfation. In sharp contrast, the methylated polyphenols, four studied here (Fig. 1) and one (3',4'-dimethoxyflavone) previously (Wen and Walle, 2006b), all displayed high stability in the human liver S9 fraction.
fraction compared with their unmethylated analogs. Their rate of disappearance was very similar, although the conditions used in our study did not permit any more detailed comparison. Their metabolic stability will obviously depend on cytochrome P450 oxidation (Wen and Walle, 2006b). This remains an issue of future investigations.

The unmethylated polyphenols, such as chrysins, resveratrol, quercetin, and apigenin, have been shown to be extensively metabolized in the Caco-2 cells, and their glucuronic acid conjugates and sulfate conjugates were effluxed by MRP2 to the apical chamber (Walle et al., 1999; Hu et al., 2003; Kaldas et al., 2003). Inasmuch as methylation protects the dietary flavonoids from extensive conjugation, the methylated flavonoids may have a higher oral absorption than their unmethylated analogs. This assumption was confirmed by using the intestinal Caco-2 cells, which exhibit a well differentiated brush border on the apical surface with tight junctions, and express typical small intestinal enzymes and transporters (Munnier et al., 1995; Boheis et al., 2001; Sambuy et al., 2005). In the Caco-2 cell monolayer transport experiments, the methylated flavonoids showed an approximately 5- to 8-fold higher rate of absorption than the corresponding unmethylated compounds (Table 2). Based on the different rates of metabolism by the liver S9 fraction above, these differences most likely are related to higher metabolic stability of the methylated flavonoids in the intestinal epithelial cell layer, although some contributions by transporters (Walle, 2004; Chen et al., 2005) cannot be excluded. It also should be noted that, whereas glucuronidation of the unmethylated compounds (Table 2). Based on the different rates of metabolism by the liver S9 fraction above, these differences most likely are related to higher metabolic stability of the methylated flavonoids in the intestinal epithelial cell layer, although some contributions by transporters (Walle, 2004; Chen et al., 2005) cannot be excluded. It also should be noted that, whereas glucuronidation of the unmethylated flavonoids in general predominated in the liver, sulfation was more important in the Caco-2 cells. This is consistent with previous observations and is dependent on tissue-specific expression of these enzymes (Glatt, 2000; Tamura et al., 2001).

Although the methylated flavonoids were derived from chemical synthesis, almost all of them are natural products present in a variety of plant species. 7-MF is found in the extract from Meliae and Rutaceae plants (Ambrozini et al., 2004), and 7,4′-DMF has been identified in fruits and leaves from neotropical nutmeg species (Cavalcante et al., 1985; Santos et al., 1996), as well as from propolis (Popravko et al., 1969). 5,7-DMF, as mentioned in a previous study, was found in the leaves of a Malaysian Piper species (Ahmad et al., 1997), and 5,7,4′-TFM is a citrus flavonoid, present also in other plants used in folk medicine (Jaipetch et al., 1983; Yenjai et al., 2004). Several of these compounds have already been described as having potent cancer chemopreventive properties at the cancer initiation stage (Wen et al., 2005; Wen and Walle, 2005, 2006a; Tsuji and Walle, 2006). In addition, their effects at the cancer promotion stage, including inhibition of cancer cell proliferation, appear to be equally potent (T. Walle, N. Ta, T. Kawamori, U. K. Walle, P. A. Tsuji, and X. Wen, unpublished).

One reason to determine the metabolism of drugs and other compounds, including dietary chemicals, is to assess whether the metabolite products have any biological activities. However, it is more important to examine the metabolic stability as well as intestinal absorption of the parent compounds in a competent biological system. For most compounds, this determines their potential biological functions in vivo (Masimirembwa et al., 2003; Mohutsky et al., 2006).

Our findings in this study, together with our preliminary observations (Wen and Walle, 2006b), suggest metabolic stability as a new and effective approach to assess human oral bioavailability of dietary polyphenols. Such an approach is needed to help evaluate the potential usefulness of these compounds in the chemoprevention of human disease. Thus, human Caco-2 cell transport, together with metabolic stability in the human liver S9 fraction, should provide a two-pronged approach with high predictability of the in vivo situation. In summary, using these methods of evaluation, the present study demonstrates that fully methylated flavonoids have dramatically higher intestinal permeability as well as higher metabolic stability than those of the unmethylated forms. These findings, together with observations of potent biological activities, provide promise of efficient in vivo chemopreventive activities of such dietary compounds.

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References


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