Degradation of Flavonoid Aglycones by Rabbit, Rat and Human Fecal Flora

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The degradation of thirteen flavonoid aglycones—wogonin, diosmetin, hesperetin, baicalein, morin, genistein, daidzein, quercetin, naringenin, luteolin, kaempferol, apigenin and neophellamuretin—were investigated in rabbit, rat and human fecal flora suspensions as well as in artificial intestinal juice, using high performance liquid chromatography. Separation were performed with a Cosmosil 5C18 AR II column by isocratic and gradient elution with 0.1% (v/v) phosphoric acid–acetonitrile as a mobile phase, and detected at 254 nm. The flow rate was 1.0 ml/min. 5,7-Dimethoxycoumarin was used as the internal standard. The result indicated that all flavonoid aglycones except baicalein, diosmetin and quercetin were quite stable in artificial intestinal juice, whereas all were degraded in rabbit, rat and human feces suspension. In rabbit feces, wogonin, diosmetin and hesperetin were less degraded, whereas neophellamuretin, apigenin, kaempferol, luteolin, and naringenin were the most extensively degraded. In rat feces, wogonin and diosmetin were least degraded, whereas kaempferol, quercetin, genistein, luteolin, naringenin and neophellamuretin were extensively degraded. As in human feces, wogonin, daidzein and diosmetin were less degraded, whereas morin, genistein, baicalein, and quercetin were extensively degraded. In conclusion, wogonin and diosmetin were among the less degraded ones for all three feces tested. The presence of a methoxy group on the A or B ring of the flavonoid seems to protect the structure from bacterial degradation.

Key words flavonoid aglycone; degradation; HPLC

Flavonoids are a major group of natural antioxidants. 1—3) Diets high in natural antioxidants are associated with reduced risks of coronary heart disease 1,4) and possibly cancer. 5,6) Flavonoids are primarily present as glycosides in fruits, vegetables, beverages and Chinese herbs. 7) The metabolism and absorption of flavonoid glycosides are not fully understood. It has been generally accepted that flavonoid glycosides reach the large intestine, where they are hydrolyzed to absorbable aglycones which are further degraded by the intestinal microflora. 8) The extent and rate of degradation of flavonoid aglycones may also play a role in affecting the bioavailability of aglycones and their glycosides. This study simulated the events taking place in the large intestine and attempted to investigate the relationship between chemical structures and degradation by rabbit, rat and human fecal flora among thirteen flavonoid aglycones.

MATERIALS AND METHODS

Chemicals Morin, quercetin, apigenin, naringenin, hesperetin, kaempferol, genistein and 5,7-dimethoxycoumarin were purchased from Sigma (St. Louis, MO, U.S.A.). Daidzein, luteolin and diosmetin were obtained from Extrasynthese (Genay, France). Baicalein was purchased from Aldrich Chem. Co. (St. Louis, MO, U.S.A.). Wogonin and potassium dihydrogenphosphate (6.8 g) was dissolved in 250 ml water, then 190 ml of 0.2 N sodium hydroxide and 400 ml water were added and mixed well. The solution was adjusted to pH

5 7 8

R1 R2 R3 R4 R5 R6
Morin OH H OH H OH H H
Luteolin H OH OH H H H H
Quercetin H OH OH H H H H
Apigenin H H OH H H H H
Diosmetin H H OCH3 OH H H
Kaempferol H H OH H OH H H
Baicalein H H H H H OH H
Wogonin H H H H H H OH CH3

5 7 8

R1 R2 R3 R4
Naringenin OH H H H
Hesperetin OCH3 OH H H
Neophellamuretin OH H OH CH3 CH3

5 7 8

R1 R2
Daidzein H OH
Genistein H OH

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7.5 ± 0.1 with 0.2 N sodium hydroxide, then sufficient water was added to create a final volume of 1000 ml.

Preparation of Feces Suspensions The fresh feces were collected from New Zealand white rabbits and Sprague-Dawley rats in the animal center of China Medical College. Human feces were obtained from three healthy volunteers and well mixed. Sixty grams of feces were weighed, and 180 ml artificial intestinal juice (pH 7.5) was added. The mixture was homogenized using an agitator, and the homogenate was filtered through a gauze filter.

Preparation of Standard Solutions Wogonin, diosmetin, hesperetin, baicalein, morin, genistein, daidzein, quercetin, naringenin, luteolin, kaempferol, apigenin and neophyllamuretin were accurately weighed and dissolved in methanol to give a concentration of 25.0 μg/ml. All the compounds were accurately weighed and dissolved in methanol to give a concentration of 250ind ml/blank, respectively. 5,7-Dimethoxycoumarin as an internal standard was dissolved in ethyl acetate to give a concentration of 25.0 μg/ml.

Fermentation of Flavonoid Aglycones The rabbit, rat and human feces suspensions (9.72 ml) were spiked individually with various flavonoid standard solutions (1.08 ml) and were well mixed with stirring in beakers. Each aliquot of 600 μl was placed in an amber glass tube, sealed with a septum, and air was removed with a syringe. All samples were prepared in triplicate and kept at 0–4 °C before incubation. The tubes were then incubated in a shaking water bath (100 rpm) at 37 °C for 0, 1, 4, 8 and 24 h, and then samples were stored at −20 °C until analysis. In addition, the flavonoids were incubated in artificial intestinal juice, and then subjected to the same procedures as for the feces suspensions described above.

HPLC Analysis of the Flavonoids after Incubation After thawing, each sample was acidified with 100 μl of 0.1 N hydrochloric acid, then partitioned with 700 μl ethyl acetate solution (containing 5,7-dimethoxycoumarin 25.0 μg/ml). Then, the mixture was centrifuged at 9860g for 15 min and the supernatant was blown with N2 gas until dryness. The residue was reconstituted with 50 μl methanol, and 10 μl was subjected to HPLC analysis.

The HPLC system consisted of a Hitachi Model L-6200 intelligent pump and an L-3000 photo diode array detector, equipped with a Shimadzu SIL-9A autosampler. A Cosmosil 5C18-AR II column (5 μm, 150 × 4.6 mm) for rabbit, rat feces and artificial intestinal juice; 250 × 4.6 mm for human feces) with a guard column (MetaGuard 4.6 mm Polaris 5 μ C18-A, MetaChem, Torrance, CA, U.S.A.) was employed. The detector wavelength was set at 254 nm and the flow rate was 1.0 ml/min. The mobile phases used for the analysis of various samples were mixtures of 0.1% (v/v) phosphoric acid (A) and acetonitrile (B) with isocratic or gradient elution as follows:

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>ratio of mobile phase (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial intestinal juice</td>
<td>68/32</td>
</tr>
<tr>
<td>Rabbit feces</td>
<td>68/32</td>
</tr>
<tr>
<td>Rat feces</td>
<td>72/28</td>
</tr>
<tr>
<td>Human feces</td>
<td>0–5 min, 72/28; 15 min, 76/24; 30 min, 65/35; 40 min, 72/28; 50 min, 60/40; 60 min, 72/28</td>
</tr>
</tbody>
</table>

Data Analysis The percentage remained after the incubation of flavonoids for different durations was calculated from their peak area ratios (flavonoid to internal standard) as compared with those before incubation. Degradation slopes were calculated by the linear regression of the natural log of the remaining percentage (%).

RESULTS AND DISCUSSION

In this study, HPLC methods were developed for the determination of thirteen flavonoid aglycones in rabbit, rat and human feces suspension, as well as in artificial intestinal juice. The HPLC condition for the analysis of rabbit feces suspension and artificial intestinal juice was not suitable for rat and human feces. Another isocratic elution was established for rat feces. As for human feces, satisfactory separation was achieved using gradient elution. The flavonoids and internal standard were successfully separated within half an hour in rabbit feces and artificial intestinal juice, whereas rat and human feces needed 1 h for satisfactory separation. The degradation of thirteen flavonoids was investigated one-by-one in various feces in this study. Figure 1 shows chromatogram of a mixture of thirteen flavonoid standards eluted by the mobile phase for samples of human feces.

The structure of the flavonoid aglycone is characterized by a 2-phenylbenzo-γ-pyrone modified by the substitution patterns of the two aromatic benzene rings A and B, and in the oxygen-containing heterocyclic C ring. From the retention times on the HPLC chromatogram, the relationship between the polarity and structures of flavonoids could be proposed. Among the thirteen flavonoids, morin is most polar. As a positional isomer of quercetin, the meta-disposed dihydroxy groups make the molecule of morin more polar than the ortho-disposed quercetin. Naringenin and hesperetin are slightly more polar than their correspondent flavones, i.e. apigenin and diosmetin, respectively. Quercetin is the 3-hydroxy derivative of luteolin, but luteolin showed slightly higher polarity than quercetin, indicating that the presence of a 3-hydroxy group decreased the polarity. Genistein is the 5-hydroxy derivative of daidzein, but daidzein showed slightly higher polarity than genistein, indicating that the presence of 5-hydroxy group decreased the polarity. It could be specu-

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Fig. 1. HPLC Chromatogram of Flavonoid Standards

1, morin; 2, daidzein; 3, luteolin; 4, quercetin; 5, genistein; 6, naringenin; 7, apigenin; 8, hesperetin; 9, diosmetin; 10, kaempferol; 11, baicalein; 12, 5,7-dimethoxy-coumarin (1.8); 13, neophyllamuretin; 14, wogonin.
lated that 3- or 5-hydroxy groups which formed intramolecular hydrogen bonds with the 4-one group did not increase the polarity of flavonoids, but rather, the polarity was decreased. Baicalein and wogonin possessing trihydroxy groups on the A ring and no substitution on the B ring were much less polar than other flavonoids. Wogonin with a methoxy group A ring and no substitution on the B ring were much less polar than other flavonoids, but rather, the polarity was decreased. The substitution patterns of flavonoid aglycones may significantly affect their bioavailabilities.

Figures 2—5 depict the degradation of various flavonoid aglycones in rat, rabbit and human feces, respectively. All flavonoids tested in the present study were significantly degraded in rabbit, rat and human feces suspensions, are shown in Table 1. The results indicated that genistein and daidzein seem the most stable among the thirteen flavonoids in artificial intestinal juice, indicating that the isoprenyl side chain greatly increased the lipophilicity.

The polarity of flavonoid aglycones is an important property for their bioavailability because the dissolution in gastrointestinal juice and the permeation through enterocytes of a compound are closely associated with their polarity. In general, the more polar the compound, the better the dissolution. However, too polar a compound cannot penetrate through enterocytes to enter the blood circulation. Therefore, medium polarity would be better for absorption. Flavonoid aglycones are a class of compounds with a wide spectrum of polarity which highly depends on the substitution of the three rings. The substitution patterns of flavonoid aglycones may significantly affect their bioavailabilities.

Chinese herbs containing a variety of flavonoid glycosides are generally prepared by boiling them with water in which flavonoid glycosides are quite soluble. However, flavonoid glycosides are too polar to be absorbed. Nevertheless, when the glycosides migrated down to the large intestine, the enterobacteria residing in the colon could release enzymes to cleave the sugar moieties to transform the glycosides into aglycones which were less polar and became absorbable. However, the flavonoid aglycones were further degraded into small molecules by the enterobacteria.9) The degradation rates of flavonoids in artificial intestinal juice, and in rabbit, rat and human feces suspensions, are shown in Table 1. The results indicated that genistein and daidzein seem the most stable among the thirteen flavonoids in artificial intestinal juice, indicating that isoflavones lacking a highly conjugated 4’-OH with a 4-one group were more stable than flavones. The other flavonoids were quite stable except for baicalein, diosmetin and quercetin, which all possess catechol-type structures. Baicalein was the least stable one, and its instability is explainable in that the catechol structure of the A ring is prone to be oxidized to dione, especially in basic solution. Quercetin has a B ring with a catechol structure and is like-wise as unstable as baicalein.

Figures 2—5 depict the degradation of various flavonoid aglycones in rat, rabbit and human feces, respectively. All flavonoids tested in the present study were significantly degraded in rabbit, rat and human feces suspensions. This observation was essentially in agreement with previous reports.8,9) In rabbit feces, wogonin, diosmetin and hesperetin were less degraded, whereas neophyllamuretin, apigenin, and...
kaempferol, luteolin, naringenin and quercetin were extensively degraded. In rat feces, wogonin and diosmetin were less degraded, whereas kaempferol, quercetin, genistein, luteolin and naringenin were extensively degraded. As in human feces, wogonin, daidzein and diosmetin were less degraded, whereas morin, genistein, baicalein, and quercetin were extensively degraded. Because of the shortage of neophyllamuretin, the degradation was not investigated in human feces. By comparing the degradation of flavonoids among feces of three species, very close resemblance was found between rabbits and rats, whereas human feces showed a relatively large difference, especially in terms of those flavonoids.
degraded most extensively. These results indicated that similar strains of enterobacteria might reside in rabbits and rats, whereas those in humans seemed quite different. Wogonin and diosmetin were less degraded for all species, indicating that the presence of a methoxy group on the A or B ring could protect the structure from degradation. The least polar wogonin seemed the one most resistant to bacterial degradation. Baicalein was the least stable one in artificial intestinal juice, whereas it was not the most extensively degraded in various feces, indicating that the enterobacterial degradation of many other flavonoids was superior to baicalin.

Table 1 shows the linear regression of the remaining percentages of flavonoids against incubation time. The coefficients of correlation indicated that the degradation essentially followed first order kinetics. By comparing the degradation between hesperetin and diosmetin, naringenin and apigenin, respectively, it showed whether there was a C_{2,3} double bond in the C ring did not result in a significant difference in the degradation in various feces. A previous study proposed that the C-ring of flavonols was labile for bacterial degradation to yield phenolic acids and ethylbenzene. Comparisons between the degradation of quercetin with luteolin, kaempferol with apigenin, respectively, indicated that the presence of a 3-hydroxy group did not significantly influence the ease of degradation. The isoprenyl flavonoid neophyllamuretin appeared to be quickly degraded, especially by rabbit fecal flora. Our previous pharmacokinetic study showed that the absorption of its glycoside, phyllamurin, was quite good in comparison with other glycosides, such as rutin, indicating that if the hydrolyzed aglycone is lipophilic enough, it can easily partition into enterocytes and escape from further degradation by the microflora in gut lumen.

From this study, it can be concluded that all the flavonoids tested were degraded significantly by rabbit, rat and human feces. It was observed that the structures with methoxy groups on ring A or B are relatively nonpolar, and may protect them significantly from extensive bacterial degradation. It is speculated that a flavonoid glycoside possessing a methoxy group could be beneficial for the absorption if the dissolution is satisfactory.

REFERENCES