JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

*J. Agric. Food Chem.* **2009,** *57,* 83–89 **83**

# Metabolism and Pharmacokinetics of 3,3′,4′,7-Tetrahydroxyflavone (Fisetin), 5-Hydroxyflavone, and 7-Hydroxyflavone and Antihemolysis Effects of Fisetin and Its Serum Metabolites

CHI-SHENG SHIA,[†] SHANG-YUAN TSAI,[‡] SHENG-CHU KUO,[†] YU-CHI HOU,*[,‡] AND PEI-DAWN LEE CHAO*[,‡]

Institute of Pharmaceutical Chemistry and School of Pharmacy, China Medical University, Taichung 40402, Taiwan

3,3′,4′,7-Tetrahydroxyflavone (fisetin) has shown various beneficial bioactivities. This study investigated the metabolism and pharmacokinetics of fisetin, 5-hydroxyflavone (5-OH-flavone), and 7-hydroxyflavone (7-OH-flavone) in male Sprague−Dawley rats. Blood was withdrawn via cardiopuncture and assayed by HPLC before and after hydrolysis with sulfatase and $\beta$-glucuronidase. The results indicated that after intravenous administration of fisetin (10 mg/kg of bw), fisetin declined rapidly and fisetin sulfates/glucuronides emerged instantaneously. When fisetin (50 mg/kg of bw) was given orally, fisetin parent form was transiently present in serum only during the absorption phase, whereas fisetin sulfates/glucuronides predominated. The serum metabolites of fisetin showed less potent inhibition on 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced hemolysis than fisetin. Following oral administrations of 40 mg/kg of bw of 5-OH-flavone and 7-OH-flavone, the glucuronide of 5-OH-flavone and the sulfate/glucuronide of 7-OH-flavone were found in serum, whereas no traces of parent forms were detected. In conclusion, fisetin and 7-OH-flavone were rapidly and extensively biotransformed into their sulfate/glucuronide, whereas 5-OH-flavone was exclusively metabolized to glucuronide.

**KEYWORDS: Fisetin; 3,3′,4′,7-tetrahydroxyflavone; 5-hydroxyflavone; 7-hydroxyflavone; pharmacokinetics; metabolism; sulfates; glucuronides**

## INTRODUCTION

Epidemiological studies have suggested a protective role of dietary flavonoids against coronary heart disease and possibly cancer (*1, 2*). In addition, flavonoids have attracted increasing attention in recent years because of various beneficial biological activities including anti-inflammatory, antiallergic (*3*), antiviral (*4,5*), anticancer (*6, 7*), and antioxidation properties (*8, 9*). However, there is a very large amount of in vitro data, but far fewer reports of animal studies of flavonoids are available. Whether the biological activities of flavonoids observed in vitro can be extended to human subjects remains questionable.

3,3′,4′,7-Tetrahydroxyflavone (fisetin), a rare flavone without 5-hydroxy substitution, is distributed in *Rhus cotinus* L. (*10*), *Cotinus coggygria* SCOP. (Anacardiaceae) (*11*), and *Acacia catechu* Willd. (Leguminosae) (*12*). Many studies have reported that fisetin exhibits beneficial activities including cardioprotective (*13*), anticancer (*14*), antiallergic (*15*), and antithyroid effects (*16*) as well as inhibition of angiogenesis (*17*) and glucose uptake (*18*). However, most of the findings were essentially based on in vitro studies. Over the past decade, study of the metabolic fates of flavonoids in animals has gradually recognized that flavonoid aglycones are generally not present in the circulation, whereas their glucuronides and sulfates are the principle forms (*19*). Until now, the metabolism and pharmacokinetics of fisetin have not been reported in the literature. Therefore, whether the in vitro activities of fisetin could predict the in vivo effects is an important unanswered question. Therefore, this study investigated the metabolism and pharmacokinetics of fisetin in rats. Furthermore, as an extension of our pharmacokinetic study, the serum metabolites of fisetin were prepared from rats and quantitated, and then the activities of fisetin and its serum metabolites against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced hemolysis were evaluated and compared on an equimolar basis.

5-OH-flavone, a flavone possessing a strongly hydrogen-bonded hydroxyl group, is known to be very resistant to chemical derivatization such as acetylation and methylation. To understand the metabolic fate of this 5-hydroxy group in vivo, the present study comprised pharmacokinetic study of 5-OH-

* Authors to whom correspondence should be addressed [e-mail (P.-D.L.C.) pdlee@mail.cmu.edu.tw, (Y.-C.H.) houyc@mail.cmu.edu.tw].
[†] Institute of Pharmaceutical Chemistry.
[‡] School of Pharmacy.

10.1021/jf802378q CCC: $40.75 
Published on Web 12/17/2008

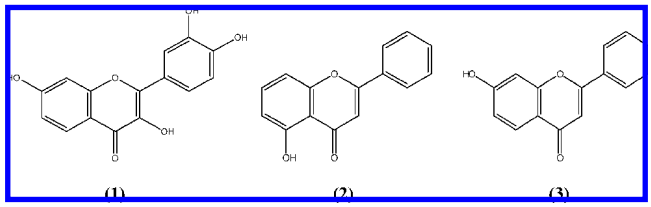


**Figure 1.** Chemical structures of fisetin (**1**), 5-OH-flavone (**2**), and 7-OH-flavone (**3**).

flavone. Additionally, the metabolic fate of 7-OH-flavone was also investigated and compared with those of 5-OH-flavone and fisetin (**Figure 1**).

## MATERIALS AND METHODS

**Materials.** Fisetin, ethyl paraben, and AAPH were purchased from Aldrich (Milwaukee, WI). Sulfatase (type H-1 from *Helix pomatia*, containing 14,000 units/g of sulfatase and 498,800 units/g of $\beta$-glucuronidase), $\beta$-glucuronidase (type B-1, from bovine liver, containing 1,240,000 units/g of $\beta$-glucuronidase), flavone, 5-OH-flavone, 7-OH-flavone, and tetraglycol were purchased from Sigma Chemical Co. (St. Louis, MO). L-(+)-Ascorbic acid was obtained from Riedel-de Haen (Seelze, Germany).

**Flavonoid Administration and Blood Collection.** Male Sprague−Dawley rats weighing 300−380 g were fasted for 15 h before flavonoid administration, and food was only supplied 3 h after dosing. Water was supplied ad libitum. For intravenous (iv) administration, fisetin was dissolved in tetraglycol and filtered through a 0.22 $\mu$m membrane filter. The iv bolii were given to six rats via the tail vein at a dose of 10 mg/kg of bw. Blood samples were withdrawn via cardiac puncture at 5, 15, 30, 45, 90, 240, 480, and 720 min postdosing.

For oral administrations, fisetin, 5-OH-flavone, 3-OH-flavone, and 7-OH-flavone were dissolved with tetraglycol and given via gastric gavage. The dose of fisetin was 50 mg/kg of bw (25 mg/mL). Blood samples were withdrawn via cardiac puncture at 5, 15, 30, 90, 180, 360, 600, 1440, 2160, and 2880 min postdosing. 5-OH-flavone and 7-OH-flavone were given at doses of 40 mg/kg of bw (20 mg/mL), which was equimolar with the fisetin oral dose. Blood samples were withdrawn at 20, 40, 60, 180, 300, 540, 720, 1440, 2160, 2880, 3600, and 4320 min after administration of 5-OH-flavone and 5, 15, 30, 60, 90, 180, 300, 540, 1440, 2160, 2880, and 4320 min after administration of 7-OH-flavone. All blood samples were centrifuged at 10000*g* for 15 min, and the serum obtained was stored at −30 °C for later analysis. The animal study adhered to *The Guidebook for the Care and Use of Laboratory Animals* (2002) (published by the Chinese Society for the

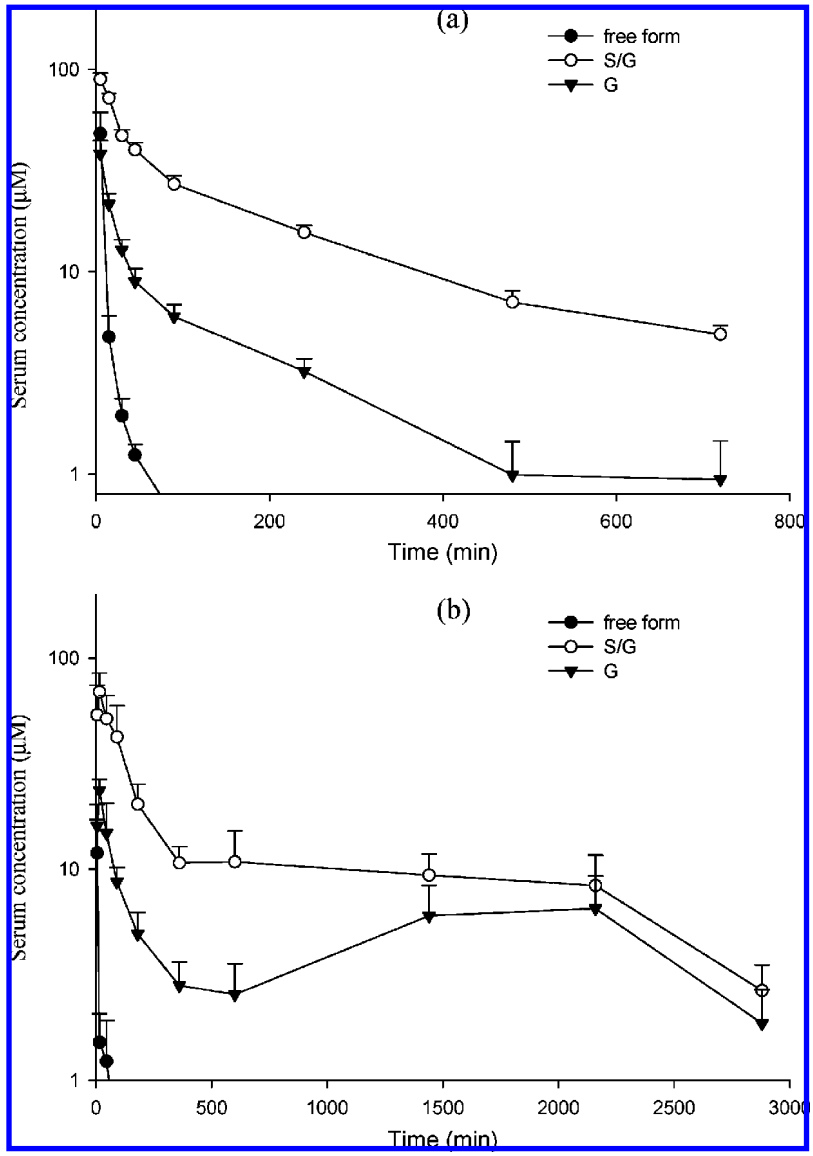


**Figure 2.** Mean (±SE) serum concentration−time profiles of fisetin (●), its sulfates/glucuronides (○), and glucuronides (▼) after intravenous administration of 10 mg/kg (**a**) and oral administration of 50 mg/kg (**b**) of fisetin to six rats.

**Table 1.** Pharmacokinetic Parameters of Fisetin Free Form, Sulfates/Glucuronides (S/G), and Glucuronides (G) after an Intravenous Bolus of Fisetin (10 mg/kg of bw) to Six Rats[a]

| parameter | free form | S/G | G |
|---|---|---|---|
| $AUC_{0-720}$ | 970.5 ± 205.9 a | 13226.9 ± 936.6 b | 2891.3 ± 580.9 ab |
| Cl | 12.8 ± 2.7 a | 0.8 ± 0.1 b | 4.3 ± 0.9 b |
| V | 289.2 ± 104.6 a | 263.1 ± 53.1 a | 738.5 ± 65.6 b |
| $t_{1/2}$ | 2.7 ± 0.3 a | 90.0 ± 10.2 b | 40.7 ± 11.6 c |

[a] Data are expressed as mean ± SE. Means in a row without a common letter differ ($P < 0.05$). $AUC_{0-720}$ (nmol·min/mL), area under serum concentration−time curve to 720 min; Cl (mL/min), clearance; *V* (mL), volume of distribution; $t_{1/2}$ (min), elimination half-life.

**Table 2.** Pharmacokinetic Parameters of Fisetin Sulfates/Glucuronides (S/G) and Glucuronides (G) after Oral Administration of Fisetin (50 mg/kg of bw) to Six Rats[a]

| parameter | S/G | G |
|---|---|---|
| $C_{max}$ | 72.1 ± 17.2 | 27.3 ± 4.0* |
| $AUC_{0-2880}$ | 31726.6 ± 5665.3 | 14355.0 ± 4008.2* |
| MRT | 1045.9 ± 148.4 | 1252.9 ± 169.8 |

[a] Data are expressed as mean ± SE (*, $P < 0.05$). $C_{max}$ (nmol/mL), peak serum concentration; $AUC_{0-2880}$ (nmol·min/mL), area under serum concentration−time curve to 2880 min; MRT (min), mean residence time.

Laboratory Animal Science, Taiwan, ROC). The protocol was reviewed and approved by the Committee of Animal Management, China Medical University, Taichung, Taiwan.

**Quantitation of Fisetin and Its Glucuronide/Sulfates in Serum.** For the quantitation of fisetin glucuronides, 100 μL of serum was mixed with 100 μL of β-glucuronidase (1000 units/mL in pH 5 acetate buffer) and 50 μL of ascorbic acid (100 mg/mL) and incubated at 37 °C for 6 h under anaerobic condition and protected from light. On the other hand, for the quantitation of fisetin sulfates, 100 μL of serum was mixed with 100 μL of sulfatase (100 units/mL in pH 5 acetate buffer) and 50 μL of ascorbic acid (100 mg/mL) and incubated at 37 °C for 4 h under anaerobic condition and protected from light. After hydrolysis, the serum was acidified with 50 μL of 0.1 N HCl and partitioned with 300 μL of ethyl acetate (containing 5 μg/mL of ethyl paraben as internal standard). The ethyl acetate layer was evaporated under $N_2$ gas to dryness and reconstituted with 50 μL of mobile phase (see the composition below), and then 20 μL was subjected to HPLC analysis. For the assay of fisetin parent form, a 100 μL serum sample was subjected to the process described above except for the addition of enzyme-free buffer. The concentration of free form was subtracted from the total fisetin in the hydrolysates to obtain the concentrations of conjugates.

For standard preparation, 100 μL of serum was spiked with various concentrations of fisetin and added to 100 μL of pH 5 buffer. The procedures followed were similar to those described above. The standard curve was plotted by linear regression of the peak area ratios (fisetin to ethyl paraben) against concentrations of fisetin.

**Quantitation of 5-OH-flavone and Its Glucuronide/Sulfate in Serum.** For the quantitation of 5-OH-flavone glucuronide, 150 μL of serum was mixed with 100 μL of β-glucuronidase (1000 units/mL in pH 5 acetate buffer) and 20 μL of ascorbic acid (200 mg/mL) and incubated at 37 °C for 4 h. On the other hand, for the quantitation of 5-OH-flavone sulfate, 150 μL of serum was mixed with 100 μL of sulfatase (1000 units/mL in pH 5 acetate buffer) and 20 μL of ascorbic acid (100 mg/mL) and incubated at 37 °C for 1 h under anaerobic condition and protected from light. After hydrolysis, the serum was partitioned with 270 μL of ethyl acetate (containing 2 μg/mL of amyl paraben as internal standard). The ethyl acetate layer was evaporated under $N_2$ gas to dryness and reconstituted with 50 μL of mobile phase (see the composition below), and 20 μL was subjected to HPLC analysis. For the assay of 5-OH-flavone parent form, 150 μL of serum sample was subjected to the process described above except for the addition of enzyme-free buffer.

For standard preparation, 150 μL of serum was spiked with various concentrations of 5-OH-flavone and added to 100 μL of pH 5 buffer. The latter procedure was the same as that described above for serum samples. The standard curve was plotted by linear regression of the peak area ratios (5-OH-flavone to amyl paraben) against concentrations of 5-OH-flavone.

**Quantitation of 7-OH-flavone and Its Glucuronide/Sulfate in Serum.** The procedures essentially followed that of 5-OH-flavone. The incubation times with β-glucuronidase and sulfatase were 2 h, as determined by a preliminary study for optimum hydrolysis. The serum was partitioned with ethyl acetate containing 0.5 μg/mL of flavone as internal standard.

**HPLC Conditions for Analysis of Fisetin, 5-OH-flavone, and 7-OH-flavone in Serum.** The HPLC apparatus included an LC-10AT pump (Shimadzu, Kyoto), an SPD-10AV UV−visible spectrophotometric detector, and an automatic injector series 200 autosampler (Perkin-Elmer). The COSMOSIL 5C18-ARII column (4.0 × 250 mm, 5 μm) was equipped with a guard column (4.6 × 50 mm, 5 μm) (GL Science Inc., Tokyo, Japan). The isocratic mobile phases consisted of acetonitrile and 0.1% phosphoric acid at ratios of 24:76, 68:32, and 57:43 (v/v), and the detection wavelengths were set at 252, 288, and 307 nm for fisetin, 5-OH-flavone, and 7-OH-flavone, respectively. The flow rate was 1.0 mL/min.

**Preparation and Quantitation of Serum Metabolites of Fisetin.** Ten Sprague−Dawley rats were fasted for 12 h before oral administration of fisetin. Blood was withdrawn at 10 min after dosing. The serum obtained was deproteinized and purified through solid phase extraction using Strata (Phenomenex, Torrance, CA). The aqueous solution was lyophilized to obtain powders and stored at −80 °C, of which an aliquot was quantitated following the procedures described above for serum assay.

**Effects of Fisetin and Its Serum Metabolites on AAPH-Induced Hemolysis.** Three Sprague−Dawley rats were fasted overnight, and blood was withdrawn and collected into vacutainer tubes containing EDTA. After removal of plasma and buffy coat, erythrocytes were washed five times with 2-fold volumes of cold phosphate-buffered saline (PBS). During the last wash, the erythrocytes were centrifuged at 3000*g* for 10 min to obtain a packed cell preparation. The packed erythrocytes were then suspended in a 4-fold volume of PBS. To 100 μL of erythrocyte suspension were added 100 μL of 100 mM AAPH (in PBS) and 200 μL of PBS containing various concentrations of fisetin, fisetin sulfates/glucuronides, or L-ascorbic acid (positive control). The reaction mixture was shaken gently and incubated at 37 °C for 3, 5, and 7 h. After incubation, an equal volume of PBS was added to the reaction mixture, which was then centrifuged at 10000*g* for 30 s. Then, 3 mL of PBS was added to 700 μL of the supernatant, and the absorbance at 540 nm was measured using a spectrophotometer (model UV-160A, Shimadzu, Kyoto, Japan). The percentages of hemolysis inhibition were calculated by the following equation:

$$\text{hemolysis inhibition (\%)} = [\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}} \times 100\% \quad (1)$$

**Data Analysis.** The pharmacokinetic parameters were analyzed by a noncompartmentalized model with the aid of the program WINNONLIN (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The peak serum concentration ($C_{max}$) was obtained from visual observation. The areas under the serum concentration−time curves ($AUC_s$) were calculated using the trapezoidal rule to the last point. Paired Student's *t* test was used for the comparison of pharmacokinetic parameters between sulfate/glucurouide and glucurouide. Data of hemolysis inhibition were statistically compared using ANOVA.

## RESULTS

Good linearity was found in the concentration range of 0.78−100.0 μg/mL ($Y = 0.177X + 0.005$, $r = 0.999$) of fisetin in serum. Validation of the analytical method indicated that all coefficients of variation and the relative errors were below 10%. The LLOQ and LOD of fisetin were 0.78 and 0.19 μg/mL, respectively. The recoveries of fisetin from serum were 96.1,

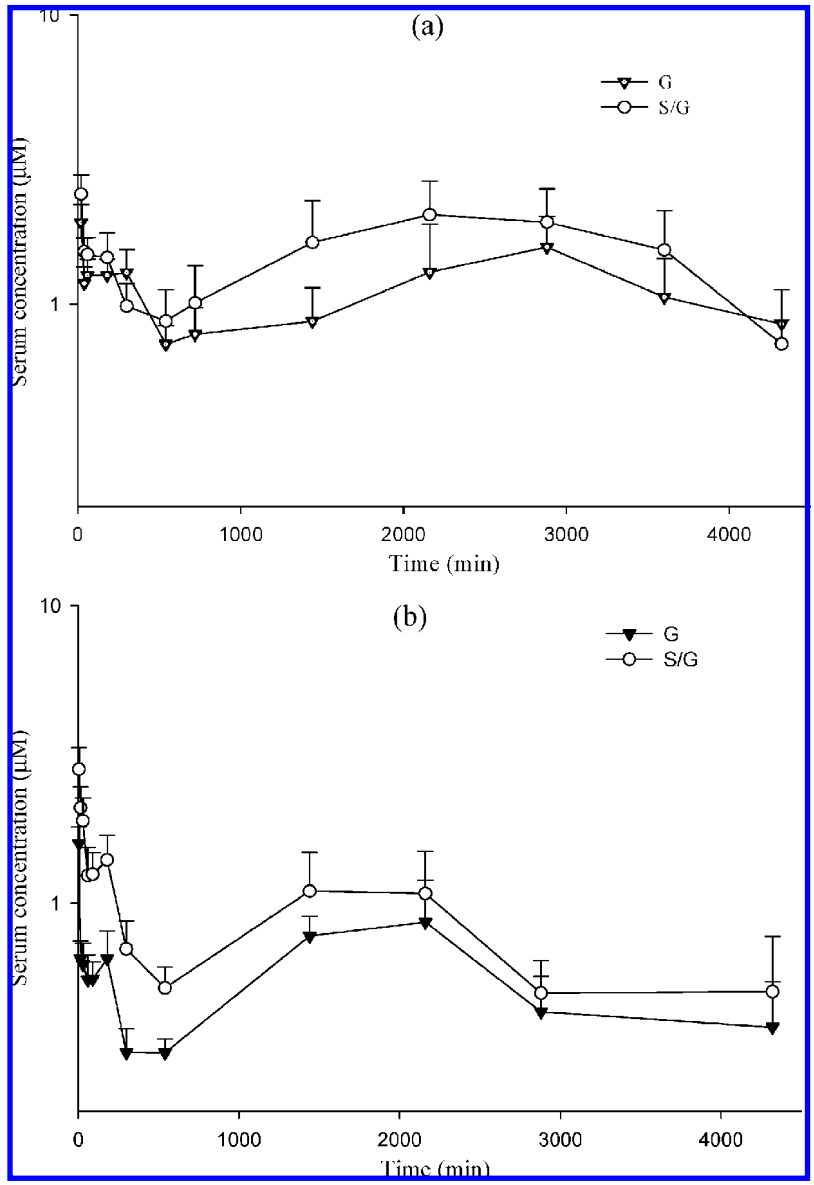


**Figure 3.** Mean (±SE) serum concentration−time profiles of sulfate/glucuronide (○) and glucuronide (▼) of 5-OH-flavone (**a**) and 7-OH-flavone (**b**) in rats after oral administration of 40 mg/kg of bw of 5-OH-flavone and 7-OH-flavone.

**Table 3.** Pharmacokinetic Parameters of Sulfate/Glucuronide (S/G) and Glucuronide (G) of 5-OH-flavone and 7-OH-flavone after Oral Administration of 40 mg/kg of bw of 5-OH-flavone and 7-OH-flavone to Six Rats[a]

| | 5-OH-flavone ($n = 6$) | | 7-OH-flavone ($n = 6$) | |
|---|---|---|---|---|
| parameter | S/G | G | S/G | G |
| $C_{max}$ | 2.7 ± 0.5 | 2.2 ± 0.5 | 2.8 ± 0.5 | 1.6 ± 0.2* |
| $AUC_{0-4320}$ | 5423.4 ± 1806.9 | 4696.8 ± 1316.4 | 3335.5 ± 555.8 | 2378.0 ± 406.8* |
| MRT | 1932.2 ± 149.7 | 2088.6 ± 66.7 | 1817.3 ± 194.5 | 1960.8 ± 173.9 |

[a] Data are expressed as mean ± SE (*, $P < 0.05$ compared with S/G of 7-OH flavone). $C_{max}$ (nmol/mL), peak serum concentration; $AUC_{0-4320}$ (nmol · min · $mL^{-1}$), area under serum concentration−time curve to 4320 min; MRT (min), mean residence time.

92.8, and 82.9% at concentrations of 3.13, 12.50, and 50.0 μg/mL, respectively. Each determination was conducted in triplicate.

Good linearity was found in the concentration range of 0.19−6.25 μg/mL ($Y = 0.89X + 0.07$, $r = 0.999$) of 5-OH-flavone in serum. Validation of the analytical method indicated that all coefficients of variation and the relative errors were below 13.5%. The LLOQ and LOD of 5-OH-flavone were 0.19 and 0.04 μg/mL, respectively. The recoveries of 5-OH-flavone from serum were 87.3, 82.7, and 85.6% at concentrations of 0.39, 1.56, and 6.25 μg/mL, respectively.

Good linearity was found in the concentration range of 0.078−1.25 μg/mL ($Y = 0.745X - 0.009$, $r = 0.999$) of 7-OH-flavone in serum. Validation of the analytical method indicated that all coefficients of variation and the relative errors were below 13.6%. The LLOQ and LOD of 7-OH-flavone were 0.078 and 0.019 μg/mL, respectively. The recoveries of 7-OH-flavone from serum were 84.0, 92.2, and 100.3% at concentrations of 0.16, 0.31, and 0.63 μg/mL, respectively.

Following an iv bolus of fisetin, the mean serum concentration−time profiles of fisetin, its sulfates/glucuronides, and glucuronides are shown in **Figure 2a**, revealing that fisetin declined rapidly; the sulfates/glucuronides and glucuronides were largely at higher concentrations than fisetin parent form at all time points. The pharmacokinetic parameters are listed in **Table 1**, indicating that the $AUC_{0-720}$ of fisetin sulfates/glucuronides was 4.6-fold that of fisetin glucuronides and 14.0-fold that of fisetin parent form.

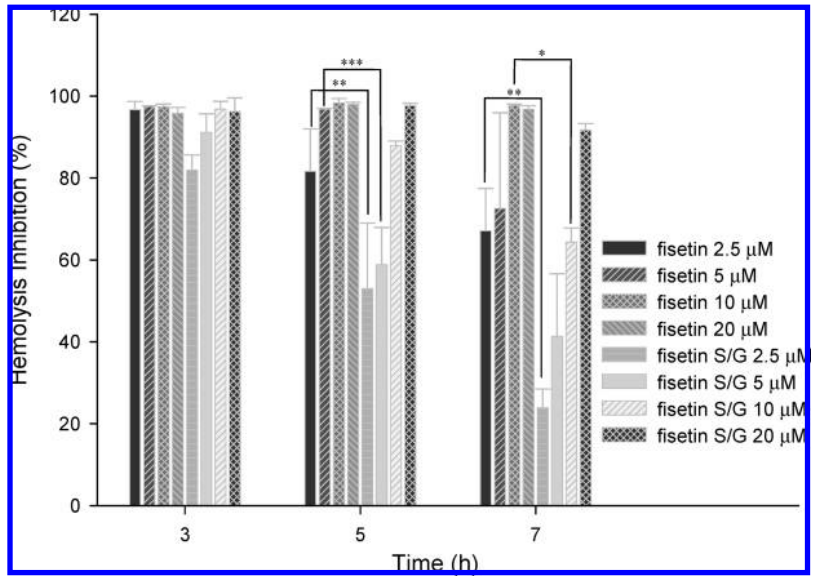


**Figure 4.** Hemolysis inhibition (percent) of 2.5, 5, 10, and 20 $\mu$M fisetin and fisetin sulfates/glucuronides (S/G) against AAPH-induced hemolysis. Data are expressed as mean ± SD of triplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

After an oral dose of fisetin, fisetin parent form could be quantitated only in a few specimens within 90 min and the $C_{max}$ was 12.2 ± 6.5 $\mu$M. After hydrolysis with sulfatase and glucuronidase, the concentrations of fisetin increased dramatically in all specimens. The mean serum profiles of fisetin, fisetin sulfates/glucuronides, and fisetin glucuronides are shown in **Figure 2b**, and the pharmacokinetic parameters are listed in **Table 2**, indicating that the $C_{max}$ and $AUC_{0-2880}$ of fisetin sulfates/glucuronides were 2.6- and 2.2-fold those of fisetin glucuronides, respectively.

Following oral administration of 40 mg/kg of bw of 5-OH-flavone and 7-OH-flavone, their parent forms were not detected in serum. **Figure 3** depicts the mean serum concentration−time profiles of the sulfate/glucuronide and glucuronide of 5-OH-flavone and 7-OH-flavone, showing that the two profiles in **Figure 3a** were largely superposable, especially the absorption phase, whereas those in **Figure 3b** diverged. The pharmacokinetic parameters are listed in **Table 3**, indicating that the $C_{max}$ and $AUC_{0-4320}$ of 5-OH-flavone sulfate/glucuronide were not significantly different from those of 5-OH-flavone glucuronide, whereas the $C_{max}$ and $AUC_{0-4320}$ of 7-OH-flavone sulfate/glucuronide were significantly higher than those of 7-OH-flavone glucuronide by 77 and 40%, respectively.

The influence of fisetin and fisetin sulfates/glucuronides on erythrocyte hemolysis was examined by incubating rat erythrocytes in the presence of 100 mM AAPH as an initiator of oxidation. The serum metabolites of fisetin have been quantitated, and the result showed equal amounts of glucuronides and sulfates contained in the mixture. During incubation for 7 h, the effects of fisetin and fisetin sulfates/glucuronides against erythrocyte hemolysis are shown in **Figure 4**, showing that both were more potent than an equivalent concentration of L-ascorbic acid (data not shown). After 7 h of incubation, 10 $\mu$M fisetin and 20 $\mu$M fisetin sulfates/glucuronides were still able to completely inhibit the hemolysis. The inhibitory effects of fisetin and fisetin sulfates/glucuronides were found to decrease in dose-dependent and time-dependent manners. The $IC_{50}$ values of fisetin and fisetin sulfates/glucuronides were 1.4 and 6.6 $\mu$M, respectively, indicating that the efficacy of fisetin sulfates/glucuronides was significantly lower than that of fisetin.

## DISCUSSION

The analytical methods of fisetin, 5-OH-flavone, and 7-OH-flavone in serum were developed and validated in this study. The precision, accuracy, and recovery were satisfactory. Owing to the commercial unavailability of the sulfates and glucuronides of various flavones, their concentrations in serum were determined indirectly through hydrolysis with sulfatase and glucuronidase. Due to the considerable amount of glucuronidase in the sulfatase (type H-1) used in this study, treatment with this enzyme resulted in the hydrolysis of both sulfates and glucuronides. Therefore, through comparison of the results between treatments with sulfatase and glucuronidase, the relative abundance of sulfates and glucuronides could be estimated. However, the concentration of double conjugates (sulfo and glucurono metabolites) of fisetin could not be determined by this method.

After an intravenous bolus of fisetin, through subtraction of the $AUC_{0-720}$ of glucuronides from that of sulfates/glucuronides, the systemic exposure of fisetin sulfates was found to be markedly higher than those of glucuronides and fisetin parent form by 260 and 1300%, respectively, indicating that fisetin was rapidly and extensively biotransformed by conjugation metabolism in liver, mainly sulfation. When fisetin was given orally, the transient presence of fisetin parent form in serum revealed the fisetin remaining after the first pass through intestine and liver. The $AUC_{0-2880}$ of fisetin sulfates/glucuronides was 2.2-fold that of fisetin glucuronides, indicating that fisetin was rapidly and extensively metabolized to sulfates and glucuronides to comparable extents, suggesting that less sulfation occurred in enterocytes than in hepatocytes when compared with the finding for the intravenous dose. In addition, the profiles near the time of peak concentration exhibiting much higher levels of fisetin sulfates/glucuronides than fisetin glucuronides indicated that fisetin was sulfated to a higher extent when absorbed in a great amount.

Following oral administrations of 5-OH-flavone and 7-OH-flavone, the parent forms were not detected in serum, indicating that the parent forms were not absorbed per se. The profiles of the sulfate/glucuronide and glucuronide of 5-OH-flavone were largely superposable, and the $C_{max}$ and $AUC_{0-4320}$ values of the sulfate/glucuronide were not significantly different from those

of glucuronide, indicating that glucuronide was the principal metabolite and sulfate was negligible. This fact revealed that the strongly hydrogen-bonded phenolic group was metabolized by glucuronidation, but not sulfation. In contrast, following the oral dose of 7-OH-flavone, the profile of the sulfate/glucuronide during the early phase was higher than that of glucuronide and both $C_{max}$ and $AUC_{0-4320}$ values of the sulfate/glucuronide were significantly higher than those of glucuronide, indicating the presence of sulfates. Our results were in good agreement with an in vitro study reporting that 7-OH-flavone was sulfated, but 5-OH-flavone was not, by human liver and duodenum (*20*). In addition, a human study has reported that chrysin (5,7-dihydroxyflavone) sulfate was the principal metabolite in plasma and that the glucuronide was too low to be determined (*21*). Taken together with our findings, it can be confirmed that the 7-OH group could be biotransformed by both sulfation and glucuronidation in vivo, whereas the 5-OH group was metabolized exclusively by glucuronidation if there was only one hydroxyl moiety in the flavone. Besides, a previous study has demonstrated that the major sites for glucuronidation are the 7-, 3-, 3′-, and 4′-hydroxyl moieties (*22*). Therefore, we suggest that both the number and position of the hydroxyl group as well as the electronic effects in the flavone structures determined the property of substrate toward glucuronidation and sulfation (*23*).

Comparison of pharmacokinetic parameters indicated that the $C_{max}$ and $AUC_{0-2880}$ valus of fisetin sulfate/glucuronide following 50 mg/kg of bw of fisetin were 27- and 59-fold the $C_{max}$ and $AUC_{0-4320}$ values of 5-OH-flavone sulfate/glucuronide, respectively, following 40 mg/kg of bw of 5-OH-flavone. In addition, the $AUC_{0-4320}$ of 7-OH-flavone sulfate/glucuronide was found to be even lower than that of 5-OH-flavone sulfate/glucuronide after an equal dose. Although three test compounds were dissolved with tetraglycol in this study and administered as clear solutions at the same molar dose, it appeared that 5-OH-flavone and 7-OH-flavone were markedly less bioavailable than fisetin. These large differences in $C_{max}$ and $AUC_{0-t}$ values can be attributed to different water solubilities between compounds. Apparently, fisetin, having four phenolic groups, is more soluble in gastrointestinal juice than 5-OH-flavone and 7-OH-flavone. We speculate that the higher solubility of fisetin may lead to better absorption and cause transient saturation of the conjugation metabolism to result in the presence of fisetin parent form during the absorption phase.

The peroxidation of erythrocyte membrane and hemolysis can be induced by various agents such as hydrogen peroxide, dialuric acid, xanthine oxidase, organic hydroperoxides, and AAPH (*24*, *25*). Our results show that both fisetin and its serum metabolites exhibited significant inhibition effects on AAPH-induced hemolysis, suggesting that fisetin sulfates/glucuronides retain free radical scavenging activity, which can be attributed to the presence of residual phenolic groups after conjugation metabolism. The fact that the antihemolysis activity of fisetin sulfates/glucuronides was significantly weaker than that of fisetin can be accounted for by the conjugation metabolism toward the catechol moiety, which is a potential antioxidant structure.

Despite the voluminous in vitro bioactivity studies reporting various beneficial effects of flavonoids, our findings of the transient presence of fisetin and the absence of 5-OH-flavone and 7-OH-flavone in serum suggest that it is difficult to infer the in vivo effects from their in vitro activities. In fact, the principal metabolites were glucuronides and sulfates, which possess very different physicochemical properties from their parent forms. These metabolites should play more important roles in in vivo activities than their parent forms. Several recent studies found the sulfates/glucuronides of morin and quercetin showed more promising bioactivities than their free forms (*26*, *27*), pointing to the possibility that the conjugated metabolites of polyphenols were not necessarily inactive and might be the principal active forms. Probably owing to the commercial unavailability of flavonoid metabolites, most in vitro bioactivity studies of flavonoids focused on the parent forms of aglycones. It is suggested that biologists pay attention to the conjugated metabolites of flavonoids. However, $\beta$-glucuronidase was found to be present in liver and increasingly released by neutrophils at inflammation sites (*28*, *29*). Therefore, deglucuronidation might occur in various tissues, resulting in the formation of flavonoid aglycones, which warrant further studies.

In conclusion, fisetin and 7-OH-flavone were rapidly and extensively transformed to sulfates and glucuronides, whereas 5-OH-flavone was exclusively metabolized to glucuronide. The serum metabolites of fisetin exerted lower potency than fisetin on hemolysis protection.

## ABBREVIATIONS USED

5-OH-flavone, 5-hydroxyflavone; 7-OH-flavone, 7-hydroxyflavone; HPLC, high-performance liquid chromatography; iv, intravenous; S/G, sulfate/glucuronide; G, glucuronide; $C_{max}$, peak serum concentration; $t_{max}$, time to peak concentration; AUCs, areas under the curves; Cl, clearance; *V*, volume of distribution; $t_{1/2}$, elimination half-life; MRT, mean residence time; PBS, phosphate-buffered saline; AAPH, 2,2′-azobis(2-amidinopropane hydrochloride).

## LITERATURE CITED

(1) Hertog, M. G.; Feskens, E. J.; Hollman, P. C.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007–1011.

(2) Graf, B. A.; Milbury, P. E.; Blumberg, J. B. Flavonols, flavones, flavanones, and human health: epidemiological evidence. *J. Med. Food* **2005**, *8*, 281–290.

(3) Middleton, E., Jr. Effect of plant flavonoids on immune and inflammatory cell function. *Adv. Exp. Med. Biol.* **1998**, *439*, 175–182.

(4) Wang, H. K.; Xia, Y.; Yang, Z. Y.; Natschke, S. L.; Lee, K. H. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. *Adv. Exp. Med. Biol.* **1998**, *439*, 191–225.

(5) Kaul, T. N.; Middleton, E., Jr.; Ogra, P. L. Antiviral effect of flavonoids on human viruses. *J. Med. Virol.* **1985**, *15*, 71–79.

(6) Yang, C. S.; Lambert, J. D.; Hou, Z.; Ju, J.; Lu, G.; Hao, X. Molecular targets for the cancer preventive activity of tea polyphenols. *Mol. Carcinog.* **2006**, *45*, 431–435.

(7) Adhami, V. M.; Mukhtar, H. Polyphenols from green tea and pomegranate for prevention of prostate cancer. *Free Radical Res.* **2006**, *40*, 1095–1104.

(8) de Groot, H.; Rauen, U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharmacol.* **1998**, *12*, 249–255.

(9) Seifried, H. E.; Anderson, D. E.; Fisher, E. I.; Milner, J. A. A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.* **2007**, *18*, 567–579.

(10) Sando, C. E.; Bartlett, H. H. The flavones of *Rhus*. *Am. J. Bot.* **1918**, *5*, 112–119.

(11) Duke, J. A. *Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants*; CRC Press: Boca Raton, FL, 1992; p 3.

(12) Li, T. S. C. *Chinese and Related North American Herbs: Phytopharmacology and Therapeutic Values*; CRC Press: Boca Raton, FL, 2002; p 413.

(13) Taubert, D.; Berkels, R.; Klaus, W.; Roesen, R. Nitric oxide formation and corresponding relaxation of porcine coronary arteries induced by plant phenols: essential structural features. *J. Cardiovasc. Pharmacol*. **2002**, *40*, 701–713.
(14) Chen, Y. C.; Shen, S. C.; Lee, W. R.; Lin, H. Y.; Ko, C. H.; Shih, C. M.; Yang, L. L. Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. *Arch. Toxicol*. **2002**, *76*, 351–359.
(15) Cheong, H.; Ryu, S. Y.; Oak, M. H.; Cheon, S. H.; Yoo, G. S.; Kim, K. M. Studies of structure activity relationship of flavonoids for the anti-allergic actions. *Arch. Pharm. Res*. **1998**, *21*, 478–480.
(16) Ferreira, A. C. F.; Lisboa, P. C.; Oliveira, K. J.; Lima, L. P.; Barros, I. A.; Carvalho, D. P. Inhibition of thyroid type 1 deiodinase activity by flavonoids. *Food Chem. Toxicol*. **2002**, *40*, 913–917.
(17) Fotsis, T.; Pepper, M. S.; Montesano, R.; Aktas, E.; Breit, S.; Schweigerer, L.; Rasku, S.; Wahala, K.; Adlercreutz, H. Phytoestrogens and inhibition of angiogenesis. *Baillieres Clin. Endocrinol. Metab*. **1998**, *12*, 649–666.
(18) Park, J. B. Flavonoids are potential inhibitors of glucose uptake in U937 cells. *Biochem. Biophys. Res. Commun*. **1999**, *260*, 568–574.
(19) Chao, P. D. L.; Hsiu, S. L.; Hou, Y. C. Flavonoids in herbs: biological fates and potential interactions with xenobiotics. *J. Food Drug Anal*. **2002**, *10*, 219–228.
(20) Vietri, M.; Pietrabissa, A.; Spisni, R.; Mosca, F.; Pacifici, G. M. 7-OH-flavone is sulfated in the human liver and duodenum, whereas 5-OH-flavone and 3-OH-flavone are potent inhibitors of SULT1A1 activity and 7-OH-flavone sulfation rate. *Xenobiotica* **2002**, *32*, 563–571.
(21) Walle, T.; Otake, Y.; Brubaker, J. A.; Walle, U. K.; Halushka, P. V. Disposition and metabolism of the flavonoid chrysin in normal volunteers. *Br. J. Clin. Pharmacol*. **2001**, *51*, 143–146.
(22) Davis, B. D.; Brodbelt, J. S. Regioselectivity of human UDP-glucuronosyl-transferase 1A1 in the synthesis of flavonoid glucuronides determined by metal complexation and tandem mass spectrometry. *J. Am. Soc. Mass Spectrom*. **2008**, *19*, 246–256.
(23) Zhang, L.; Lin, G.; Zuo, Z. Position preference on glucuronidation of mono-hydroxylflavones in human intestine. *Life Sci*. **2006**, *78*, 2772–2780.
(24) Miki, M.; Tamai, H.; Mino, M.; Yamamoto, Y.; Niki, E. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by alpha-tocopherol. *Arch. Biochem. Biophys*. **1987**, *258*, 373–380.
(25) Hatherill, J. R.; Till, G. O.; Ward, P. A. Mechanisms of oxidant-induced changes in erythrocytes. *Inflammation Res*. **1991**, *32*, 351–358.
(26) Fang, S. H.; Hou, Y. C.; Chang, W. C.; Hsiu, S. L.; Chao, P. D. L.; Chiang, B. L. Morin sulfates/glucuronides exert anti-inflammatory activity on activated macrophages and decreased the incidence of septic shock. *Life Sci*. **2003**, *74*, 743–756.
(27) Hsieh, C. L.; Chao, P. D. L.; Fang, S. H. Morin sulphates/glucuronides enhance macrophage function in microgravity culture system. *Eur. J. Clin. Invest*. **2005**, *35*, 591–596.
(28) O'Leary, K. A.; Day, A. J.; Needs, P. W.; Mellon, F. A.; O'Brien, N. M.; Williamson, G. Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human $\beta$-glucuronidase, sulfotransferase, catechol-*O*-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. *Biochem. Pharmacol*. **2003**, *65*, 479–491.
(29) Shimoi, K.; Nakayama, T. Glucuronidase deconjugation in inflammation. *Methods Enzymol*. **2005**, *400*, 263–272.

**Received for review April 18, 2008. Revised manuscript received November 10, 2008. Accepted November 10, 2008. This study was partly supported by the National Science Council (NSC95-2320-B039-023-MY2, NSC 93-2320-B-039-020) Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, ROC (CCMP96-RD-019) and the China Medical University (CMU95-087).**

JF802378Q