Determinations of Morin, Quercetin and Their Conjugate Metabolites in Serum

Su-Lan HSU, a Chih-Wan TSAO, b Yu-Chuan TSAI, b Hong-Jing HO, c and Pei-Dawn Lee CHAO*. a

School of Pharmacy, a Graduate Institute of Pharmaceutical Chemistry, b and Graduate Institute of Chinese Pharmaceutical Sciences, c China Medical College, Taichung, Taiwan, 404 R.O.C. Received January 5, 2001; accepted April 18, 2001

Morin and quercetin are isomeric antioxidant flavonols. High-performance liquid chromatographic methods were developed for the quantitation of morin and quercetin in serum. The method employed a Cosmosil RP-18 column, using acetonitrile/0.2% o-phosphoric acid: 28:72 and 27:73 (v/v) as mobile phases, with ethyl paraben and 6,7-dimethoxycoumarin used as internal standards for morin and quercetin, respectively. Moreover, a strategy to stabilize morin/quercetin released from their glucuronides/sulfates in serum during hydrolysis was established. The present methods are applicable for determining morin, quercetin, and their glucuronides/sulfates in serum.

Key words morin; quercetin; serum; HPLC

Morin (2’3,4’,5,7-pentahydroxyflavone) and quercetin (3,3’,4’,5,7-pentahydroxyflavone) are isomeric antioxidant flavonols1,2 widely distributed in fruits and vegetables as well as in many Chinese herbs. It has been reported that morin has numerous many pharmacologic effects such as preventing coronary artery disease, inhibiting proliferation of tumors3,4 and protecting human erythrocytes, ventricular tumors3,4 and protecting human erythrocytes, ventricular and saphenous vein endothelial cells,5 as well as free radical scavenging activity.6 Quercetin is a potential drug of the future because it has a variety of beneficial pharmacologic effects. It has been found to inhibit the growth of cancer cells in vitro,7 and to reduce tumor development in experimental animals,8 as well as to inhibit the induction and progression of human cancer,9,10 which made it a candidate for phase I evaluation in cancer patients.11

Flavonoids have been of great interest in recent years, however, limited information are available for flavonoid absorption. There is growing evidence that glucuronidation is central to flavonoid metabolism and absorption.12 In this study, high-performance liquid chromatographic methods were developed for the determination of morin, quercetin, and their glucuronides/sulfates in rabbit serum. Moreover, a strategy was established to protect morin or quercetin from decomposition when they are released from their glucuronides/sulfates during hydrolysis.

MATERIALS AND METHODS

Chemicals Morin, quercetin dihydrate, glycofurol, acetic acid (99%) and β-glucuronidase (from Helix pomatia containing 89400 units/ml β-glucuronidase and 3300 units/ml sulfatase) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile (LC grade), ethyl acetate (LC grade), and methyl alcohol (LC grade) were obtained from Mallinckrodt Baker, Inc. (U.S.A.), and potassium dihydrogen phosphate was the product of Merck (Germany). t(+) Ascorbic acid was purchased from Riedel-deHaen AG (Germany). Ethyl paraben was purchased from Aldrich (Milwaukee, WI, U.S.A.). Milli-Q plus water (Millipore, Bedford, MA, U.S.A.) was used for all preparations.

Instrumentation and HPLC Conditions The HPLC apparatus included two pumps (LC-6AD, Shimadzu, Japan), an SLC-6B controller, an UV spectrophotometric detector (SPD-6A, Shimadzu, Japan), and a chromatopac (C-R6A, Shimadzu, Japan). The RP-18 column (Cosmosil, 250×4.6 mm) was equipped with a prefilter. The mobile phase was acetonitrile–0.2% o-phosphoric acid (28:72 and 27:73 [v/v] for morin and quercetin, respectively) and the flow rate was 1.0 ml/min with the detection wavelength set at 250 nm and 370 nm, respectively.

Shaker ZC4000 was provided by Deng Yng (Taichung, Taiwan). Nitrogen evaporator (N-EvapTM 112) was supplied by Organomation Associates, Inc. (U.S.A.).

Stabilization of Quercetin Quercetin serum standards (100.0 μg/ml, 100 μl) in triplicate were incubated at 37 °C under various conditions. For the control, samples were not subjected to incubation. Group 1 incubation was conducted without any treatment. Group 2 incubation was carried out anaerobically. Group 3 incubation included ascorbic acid (300 mg/ml, 20 μl). Group 4 incubation was conducted anaerobically with the addition of ascorbic acid (300 mg/ml, 20 μl). Group 2 and group 4 samples were tightly sealed with septum. All samples were wrapped with aluminum foil, and the incubation was carried out in a reciprocating shaker at 37 °C for 4 h. After incubation, 50 μl 0.01 N HCl was added to each tube, and then 150 μl ethyl acetate (containing 60.0 μg/ml 6,7-dimethoxycoumarin as internal standard) was used...
for partitioning. The ethyl acetate was evaporated by blowing \( \text{N}_2 \) using a nitrogen evaporator, and then the residue was reconstituted with 200 \( \mu \text{l} \) \( \text{CH}_3\text{OH} \), of which 20 \( \mu \text{l} \) was subjected to HPLC analysis. The peak-area ratios of quercetin to internal standard among the control and four test groups after incubation were compared.

**Preparation of Calibration Curves** Two calibration curves were established for the quantitation of morin and quercetin in serum, respectively.

a. Morin

Various concentrations of morin stock solution (in \( \text{CH}_3\text{OH} \)) were spiked into blank serum to afford a series of serum standards consisting of 1.6, 6.3, 25.0, 50.0, 100.0, and 200.0 \( \mu \text{g/ml} \). To 200 \( \mu \text{l} \) serum standard, 100 \( \mu \text{l} \) buffer solution (pH 5) and 20 \( \mu \text{l} \) ascorbic acid (300 mg/ml) were added, followed by the addition of 300 \( \mu \text{l} \) ethyl acetate (containing ethyl paraben 5.0 \( \mu \text{g/ml} \)). The ethyl acetate layer was evaporated under \( \text{N}_2 \) and reconstituted with 20 \( \mu \text{l} \) \( \text{CH}_3\text{OH} \), and then subjected to HPLC analysis.

b. Quercetin

Various concentrations of quercetin stock solution (in \( \text{CH}_3\text{OH} \)) were spiked into blank serum to afford a series of serum standards consisting of 2.5, 1.2, 0.6, 0.3, and 0.2 \( \mu \text{g/ml} \). The subsequent procedures were the same as those for morin except 2.0 \( \mu \text{g/ml} \) 6,7-dimethoxycoumarin was used as the internal standard.

The peak-area ratios (morin to ethylparaben or quercetin to 6,7-dimethoxycoumarin) of serum standards at various concentrations were determined in duplicate. A calibration curve was drawn after linear regression of the peak-area ratios of morin or quercetin.

**Validation of Assay Method** The precision and accuracy of the method were evaluated by intra-run and inter-run assays of serum standards in triplicate. Recoveries were calculated based on the detected concentrations in serum compared with those in water.

**Animals and Drug Administration** Male New Zealand White rabbits, weighing 2—3 kg, were fasted for 24 h before drug administration. Morin or quercetin was freshly dissolved in glycofurol (70.0 mg/ml) and filtered through a 0.2 \( \mu \text{m} \) membrane.

An oral dose of morin or quercetin (50 mg/kg) was given to rabbits by gastric gavage. Blood samples were withdrawn from the left ear vein at specific time points, allowed to clot, and then centrifuged at 9860 \( \times g \) for 10 min to obtain serum that was stored at \(-30^\circ \text{C}\) until analysis.

**Determination of Morin or Quercetin and Its Glucuronides/Sulfates in Serum** For the determination of the free form morin or quercetin, 200 \( \mu \text{l} \) serum sample was processed as described for calibrators. For the determination of total amounts of morin or quercetin with its glucuronides/sulfates, serum sample (200 \( \mu \text{l} \)) was added to 100 \( \mu \text{l} \) \( \beta \)-glucuronidase (\( \beta \)-glucuronidase 110.4 units/ml, sulfatase 4.2 units/ml in pH 5 buffer) and 20 \( \mu \text{l} \) ascorbic acid (300 mg/ml). The vial was tightly sealed with a septum and wrapped with aluminum foil. Incubation was then conducted anaerobically in a water bath shaker at 37 \( ^\circ \text{C}\) for 4 h. After incubation, each tube was treated as calibrators prior to HPLC analysis.

The levels of glucuronides/sulfates of morin or quercetin in each serum sample were calculated by subtracting the free form concentration from the total morin or quercetin concentration after enzymatic hydrolysis.

**RESULTS AND DISCUSSION**

In view of the degradation of morin or quercetin during incubation at 37 \( ^\circ \text{C}\) in our preliminary tests (data not shown), strategies for protecting these polyphenolic flavonoids were studied using quercetin as an example. Ascorbic acid was added to the serum and the incubation was conducted anaerobically and protected from light to prevent the potential oxidation of quercetin released from its glucuronides/sulfates. The amount of quercetin recovered after incubation with various treatments indicated that no decay of quercetin in group 4 samples was observed when compared with the control group. The other three groups showed insufficient protection of quercetin. These results (as shown in Table 1) suggested that the stabilization of quercetin by the combined treatment with ascorbic acid and air withdrawal was satisfactory. The liquid–liquid partition using EtOAc was simply carried out in a microvial (1.5 ml) which is easier than the previous method using SPE.13 It is comparable to the method recently reported by Manach et al.14 and much simpler than the post-column derivatization methods.15 A time study indicated that the amount of quercetin released from its glucuronides/sulfates after incubation with glucuronidase/sulfatase achieved the maximum at 4 h.

Using our HPLC method, morin/quercetin was well resolved with \( \text{CH}_3\text{CN}–0.2\% \alpha\)-phosphoric acid (27:73 v/v) for morin and 28:72 v/v for quercetin, respectively) as the mobile phase with ethyl benzene/6,7-dimethoxycoumarin as the internal standard. Both morin and quercetin were rapidly eluted in 7 min. Good lineairities were shown for morin in the range of 1.6—200.0 \( \mu \text{g/ml} \) and for quercetin in the range of 0.2—2.5 \( \mu \text{g/ml} \). The intra-run and inter-run coefficients of variation for morin concentrations ranging from 200.0 to 1.6 \( \mu \text{g/ml} \) (Table 2) were 0.1—1.9% and 0.2—2.7% with the relative errors less than 10.9% and 8.9%, respectively, while those for quercetin concentrations ranging from 2.5 \( \mu \text{g/ml} \) to 0.2 \( \mu \text{g/ml} \) were 0.6—6.7% and 1.8—5.4% with the relative errors less than 6.4 and 8.9%, respectively, as shown in Table 3. Therefore the precision and accuracy were satisfactory for both compounds. The limits of quantitation were 1.6 and 0.2 \( \mu \text{g/ml} \) for morin and quercetin, respectively, whereas the limits of detection were 0.4 \( \mu \text{g/ml} \) and 4.9 ng/ml, respectively.
In summary, in our present method, morin or quercetin was well protected from decomposition during serum pretreatment. Furthermore, the HPLC methods developed were rapid and had good precision, accuracy, selectivity, and sensitivity and thus are applicable in pharmacokinetic studies of morin and quercetin or their glycoside precursors.

REFERENCES