Mass Spectrometric Identification and High-Performance Liquid Chromatographic Determination of a Flavonoid Glycoside Naringin in Human Urine

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The aim of this study was to evaluate the absorption of a citrus flavonoid, naringin, as its glycosylated form. Six healthy volunteers (three males and three females) were studied. After a single oral administeration of 500 mg of naringin, intact naringin was isolated from 2−4 h urine. Isolated naringin was identified by the LC/electrospray ionization mass spectrometry (ESI-MS), MS/MS, and MS/MS/MS techniques. The cumulative urinary excretion of naringin and its metabolites (naringenin and naringenin glucuronides) was determined by HPLC for 0−24 h. Approximately 0.02% of the administered dose was recovered in urine as unchanged naringin, whereas urinary recoveries of naringenin and naringenin glucuronides were approximately 0.4 and 3.6% of the administered dose, respectively. It was concluded that trace amounts of orally administered naringin can be absorbed as the glycoside. However, it is not clear whether the glycoside is cleaved before or after absorption to generate naringenin.

Keywords: *Flavonoid glycoside; HPLC; naringin; ESI/MS; human urine*

INTRODUCTION

Flavonoids occur naturally in the plant kingdom and are widely contained in the human diet. These flavonoids have shown many biological and pharmacological activities, such as antioxidant activity (Bors et al., 1990; Cao et al., 1997; Rice-Evans, 1995; Tikkanen et al., 1998), tumor growth-inhibitory activity in various cancer cell lines in vitro (Markaverich et al., 1988; Menon et al., 1998; Singhal et al., 1995; So et al., 1996), and reducing activity against the risk of the breast cancer (Messina et al., 1991, 1997). Most of these activities have been demonstrated by the aglycons rather than by the flavonoid glycosides themselves, although flavonoids are usually present in dietary plants in the form of glycosides. For example, phytoestrogens are commonly considered to exert their biological activities when the flavonoid glycosides are converted to their aglycons through deconjugation by bacteria in the gut (King et al., 1998).

A flavonoid glycoside naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) (Figure 1) occurrs in citrus grapefruit as a major flavonoid (Rouseff et al., 1987). Since the report by Booth et al. was published in 1958, naringin has been thought to be hydrolyzed to naringenin, presumably in the gut. It has recently been reported that after the ingestion of grapefruit juice or naringin itself, naringenin and its glucuronides were recovered from the human urine, whereas no naringin and naringin glucuronides were detected (Ameer et al., 1996; Fuhr et al., 1995). Furthermore, naringin was

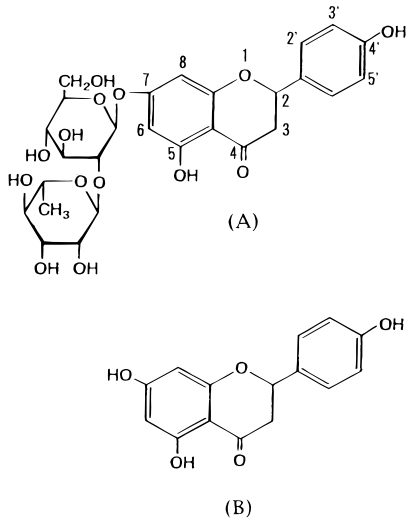

Figure 1. Structures of naringin (A) and naringenin (B).

demonstrated to be the best substrate for a human intestinal anaerobic bacterium, *Bacteroides* JY-6, producing α-L-rhamnosidase and β-D-glucosidase (Jang et al., 1996).

Naringin and several other flavonoids that possess a glycoside moiety at the 7-position of the flavonoid skeleton, such as rhoifolin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) and daidzin (4′,7-dihydroxyisoflavone-7-glucoside), show the ability to activate polymorphonuclear leukocyte (PMN) to induce the cytotoxic activity of PMN against tumor cell in vitro (Morikawa, 1990). The position and identity of the sugar moiety have been shown to be of significant importance in activating PMN. It is then possible that naringin displays the PMN activating property in vivo as its

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intact glycosylated form, if absorbed from the gut. In our preliminary experiments on naringin bioavailability, we found a peak corresponding to naringin on the HPLC chromatograms of human urine extracts, although naringin was not detectable in the blood (Ishii et al., 1996).

This paper describes the identification of urinary naringin and the 24 h cumulative urinary excretion of naringin and its metabolites in humans.

MATERIALS AND METHODS

Chemicals and Reagents. Naringin, naringenin, hesperidin (hesperetin-7-rhamnoglucoside) and hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) were purchased from Extrasynthase (HPLC grade, Genay, France). β-Glucuronidase (from *Helix pomatia*, type H-1; 348100 activity units/g) and naringinase (from *Penicillium decumbens*, 339 activity units/g) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Sample Collection and Preparation. The subjects were six healthy volunteers (three males and three females) aged between 24 and 55 years who consented to participate in single-dose studies of naringin. The subjects received 500 mg of naringin powder, which was swallowed with 100 mL of water in the morning. Urine samples were collected just before administration and 0−2, 2−4, 4−8, 8−12, 12−16, 16−20, and 20−24 h after administration. A 1.0-mL portion of each urine sample was directly used to determine naringin and naringenin. For the naringin determination, 332.5 ng of hesperidin was added to 1.0-mL aliquots of urine as internal standard. For the determination of naringenin, 650 ng of hesperetin was added to 1.0-mL aliquots of urine as internal standard. These samples were extracted with a Sep-Pak QMA cartridge as described previously (Ishii et al., 1997). For the determination of naringenin glucuronides (two isomeric monoglucuronides were detected, data not shown), 0.5-mL aliquots of urine containing 650 ng of hesperetin as internal standard were hydrolyzed by β-glucuronidase according to the method of Axelson et al. (1981). An enzyme solution (5 mL) from *H. pomatia* containing 10000 activity units in 0.2 M acetate buffer solution (pH 4.5) was added to 0.5 mL of each urine sample. The reaction mixture was incubated at 37 °C for 24 h. After 20 mL of ethanol had been added to the mixture, the solution was vortexed for 1 min and centrifuged at 3000 rpm for 5 min. The resulting supernatant was evaporated for preventing the poor recovery from the Sep-Pak cartridge. The residue was then dissolved in 3 mL of water and extracted with a Sep-Pak Accell QMA cartridge as described previously (Ishii et al., 1997).

HPLC Apparatus and Analytical Conditions. HPLC analyses were performed on a Waters Co. (Milford, MA) liquid chromatograph controlled by a model 600 controller equipped with a model 600 pump and a model 486 tunable absorbance detector. The mobile phase was degassed with a model gastorr GT-102 degasser. Data processing was carried out with a model 21 Sic chromatocorder (System Instrument, Tokyo, Japan). The HPLC system consisted of an Inertsil ODS-2 (particle size = 5 μm) column (250 × 4.6 mm i.d.) (GL Sciences, Tokyo, Japan) and a 2-cm precolumn packed with the same material. Naringin, free unconjugated naringenin, and total (free plus conjugated) naringenin obtained after the enzymatic hydrolysis were analyzed according to the method described in a previous paper (Ishii et al., 1997).

The preparative HPLC system for isolation of naringin from urine consisted of an Inertsil PREP-ODS (particle size = 10 μm) column (250 × 10 mm) and a mini-guard column (50 × 10 mm i.d.) packed with the same material. The mobile phase was acetonitrile/0.1 M ammonium acetate/glacial acetic acid (18:81:0.5, v/v), and the flow rate was 4.0 mL min^{-1}. Detection wavelength was set at 282 nm.

When the naringinase reaction was performed for the isolated urinary naringin, the mobile phase was acetonitrile/0.1 M ammonium acetate/triethylamine (25:75:0.05, v/v) and flow rate was 1.0 mL min^{-1} . Detection wavelength was set at 280 nm.

Isolation of Naringin from Human Urine. A urine sample (5 mL) was diluted with 5 mL of water and applied to a Sep-Pak Accell QMA cartridge (5 g of packing, Waters) that had previously been conditioned by washing with 30 mL of methanol and 30 mL of distilled water. The cartridge was first eluted with 1.0 mL of distilled water. After purging with air, the cartridge was eluted with 50 mL of 1 mM formic acid in methanol. After the eluate was evaporated at 40 °C, the residue was dissolved in 200 μL of the mobile phase for the preparative HPLC. The solution was subjected to preparative HPLC as described above. The fraction (∼9 mL) corresponding to the naringin peak on the chromatogram was collected. For hydrolysis by naringinase and LC/ESI-MS analysis, 15 mL of urine was treated as described above and the eluates from preparative HPLC were evaporated. The stock solution of the naringin fraction was then prepared by dissolving the residue in 10 mL of methanol.

Hydrolysis by Naringinase of Naringin Isolated from Urine. The naringinase reaction was performed according to the slightly modifed methods of Romero et al. (1985) and Habelt et al. (1983). Naringinase (40 mg, 14 activity units) was dissolved in 5 mL of 0.1 M acetate buffer (pH 3.5). The enzyme solution (100 μL) was added to 487.4 ng of naringin purified by the preparative HPLC. The reaction mixture was incubated at 37 °C for 1 h. After 400 μL of ethanol had been added to the mixture, the solution was vortexed for 30 s and centrifuged at 3000 rpm for 5 min. The resulting supernatant was evaporated to prevent peak tailing on the HPLC chromatogram. The residue was dissolved in 25 μL of ethanol with vortex mixing for 30 s, and then 25 μL of the mobile phase were added. A 20-μL portion of this solution was subjected to HPLC.

LC/ESI-MS Apparatus and Conditions. The HPLC apparatus and chromatographic conditions were appropriately designed for LC/ESI-MS, MS/MS,, and MS/MS/MS analyses. The HPLC apparatus used was a Waters Co. liquid chromatograph controlled by a model 600S controller equipped with a model 616 pump, a model 486-MS tunable absorbance detector, and a model 21 Sic chromatocorder for the data processor. The mobile phase was degassed with a Waters In-Line degasser. A Nova-Pak C_{18} column (150 × 3.9 mm, Waters) was employed. The mobile phase was methanol/10 mM ammonium acetate (60:40, v/v), and the flow rate was 0.2 mL min^{-1}. Detection wavelength was set at 280 nm. For MS analysis, the residue obtained after 1 mL (corresponding to 244 ng of naringin) of stock solution had been evaporated was dissolved in 50 μL of the mobile phase and subjected to LC/MS. For MS/MS and MS/MS/MS analyses, 4 mL (corresponding to 975 ng of naringin) of stock solution was used.

The mass spectral data were collected with a quadrupole ion trap mass spectrometer (Finnigan LCQ instrument, San Jose, CA) equipped with a heated capillary electrospray interface. The ion polarity mode was set to the positive MS, MS/MS, and MS/MS/MS mode. The sprayer needle voltage was 4.5 kV with a nebulizer gas flow set at 75% of the maximum. The temperature of the heated capillary was 220 °C. Capillary and tube lens voltages were 3 and 10 V, respectively. The total microscan was 2 microscans. The collision gas pressure was 10^{-3} Torr. The relative collision energies for the MS/MS and MS/MS/MS mode analyses were optimized at 90 and 50% of maximum setting (5 V), respectively. The MS/MS and MS/MS/MS spectra were obtained with a precursor ion isolation width of 5 *m*/*z*.

RESULTS AND DISCUSSION

The urinary excretion of naringin was investigated in six healthy volunteers who received orally 500 mg of naringin. Figure 2A shows the HPLC chromatogram of the fraction corresponding to naringin obtained by preparative HPLC. After hydrolysis by naringinase, the

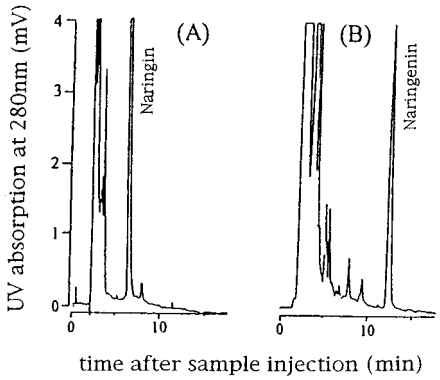

Figure 2. HPLC chromatograms of (A) naringin isolated from urine and (B) naringenin after the naringinase hydrolysis.

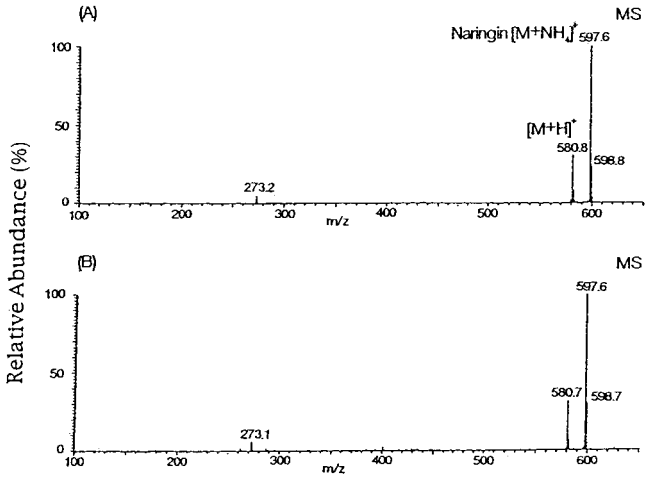

Figure 3. Positive ion ESI mass spectra: (A) MS spectrum of an authentic standard of naringin; (B) MS spectrum of naringin isolated from urine.

naringin peak disappeared and a peak corresponding to naringenin was observed as shown in Figure 2B.

The positive ion ESI mass spectrum of authentic naringin (Figure 3A) showed an abundant ion peak at *m*/*z* 598, which was the ammonium adduct ion of naringin ($[M + NH_4]^+$ [naringin, 580 atomic mass units (amu) plus 18]. When the temperature of the electrospray interface (heated capillary) was raised from 220 to 240 °C, the relative intensity of the heat-sensitive adduct ion decreased to 30% and the proton adduct ion at *m*/*z* 581 (580 amu plus 1) was obtained as the principal ion. LC/ESI-MS analysis of the naringin fraction obtained from urine yielded an intense peak at *m*/*z* 598 (Figure 3B), and the MS behavior of this peak was the same as that of the adduct ion of $[M + NH_4]^+$ from the authentic standard of naringin, giving a principal ion of *m*/*z* 581. MS/MS analysis of the *m*/*z* 598 ion from the urine sample as precursor ion gave a base peak of *m*/*z* 273 (Figure 4A), and the MS/MS/MS analysis of the *m*/*z* 598 ion then produced a single peak at *m*/*z* 153 (Figure 4B). It has already been shown that the positive chemical ionization collisionally activated dissociation tandem mass spectrometry (PCI-CAD MS/MS) of naringenin produces the proton adduct ($[M + 1]^+$) ion of *m*/*z* 273 (naringenin, 272 amu plus 1) (Ameer et al., 1996). The *m*/*z* 153 ion is a common daughter ion for flavanones and is considered to be the result of the retro-Diels−Alder reaction of the pyron ring. This ion has been used to search for naringenin, hesperetin, and related flavanones in the complex matrixes of biological

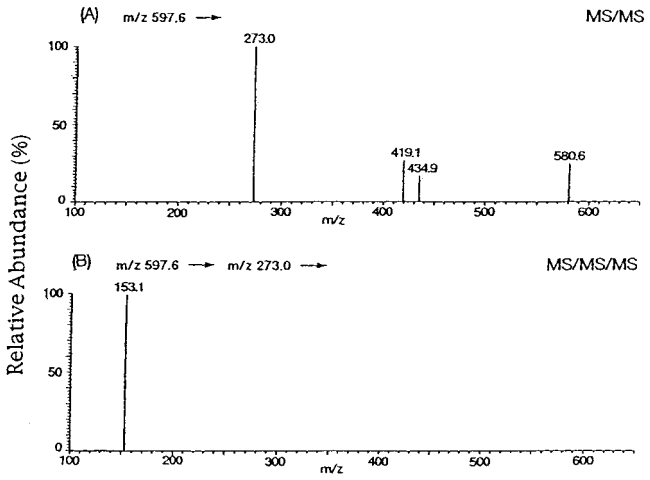

Figure 4. (A) MS/MS spectrum of the *m*/*z* 597.6 and (B) MS/MS/MS spectrum of the *m*/*z* 597.6 ion as precursor ion of naringin isolated from urine.

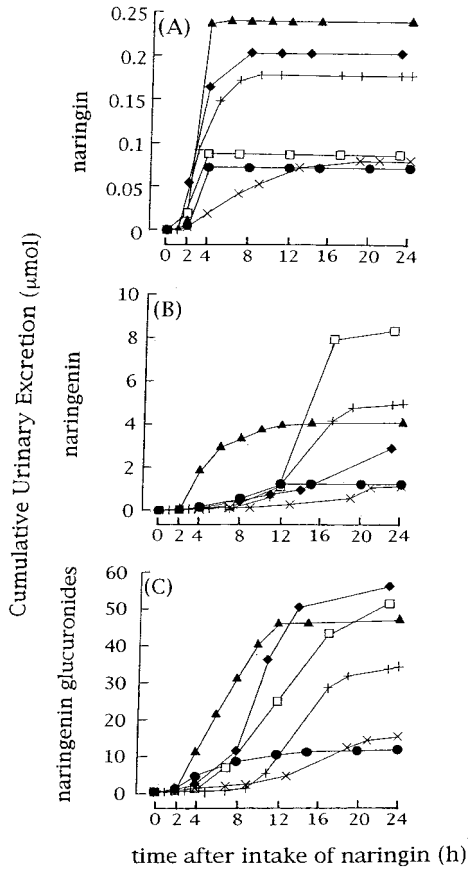

Figure 5. Cumulative urinary excretion of naringin (A), naringenin (B), and naringenin glucuronides (measured as naringenin after enzymatic hydrolysis) (C) after intake of naringin by six volunteers (□, ●, ◆, male; ▲, ×, +, female).

samples and citrus juice by the MS/MS method (Weintraub et al., 1995).

Figure 5 shows the 24-h cumulative urinary excretion of naringin, naringenin, and naringenin glucuronides after oral administration of 500 mg of naringin to six human volunteers. Naringin was absorbed as the intact glycoside. Naringin was detected in the first 0−2-h urine samples, and its cumulative excretion reached a plateau within 4−8 h except for one subject, whose cumulative amounts gradually increased up to the 16−20-h urine collection period (Figure 5A). There was a large varia-

Table 1. Urinary Recovery of Nagingin

subjects ($n = 6$)	% of nagingin administered	% of aglycon administered	
	nagingin	nagingenin	nagingenin glucuronides
median	0.02	0.380	3.559
range	0.01−0.03	0.117−0.835	1.188−5.598

tion in plateau levels among the subjects, ranging from 0.075 to 0.25 μmol (44−145 μg range).

The cumulative amounts of naringenin (Figure 5B) and its glucuronides (Figure 5C) in the 24-h urine varied to a large extent among the subjects but were comparable to published data (Ameer et al., 1996; Fuhr et al., 1995) from experiments with single doses of grapefruit juice or pure compound (500 mg of naringin). The pattern for urinary excretion of naringenin was very similar to that of naringenin glucuronides in each individual subject. The urinary recoveries of naringin, naringenin, and naringenin glucuronides were approximately 0.02, 0.4, and 3.6% of the administered dose, respectively (Table 1).

It is commonly believed that the flavonoid glycosides are not absorbed from the human gut. These glycosides are known to be biotransformed to the corresponding aglycons and sugar moieties by enteral microorganisms. Our results suggest that orally administered naringin can be absorbed from the human gastrointestinal tract as the glycoside, although trace amounts of orally administered naringin were excreted into urine as naringin itself. However, the aglycon is also absorbable, but it is not clear whether the glycoside is cleaved before or after absorption to generate naringenin.

To our knowledge, this is the first paper to provide evidence for oral absorption of naringin as the intact glycoside.

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