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Determination of naringenin and its glucuronide conjugate in rat plasma and brain tissue by high-performance liquid chromatography

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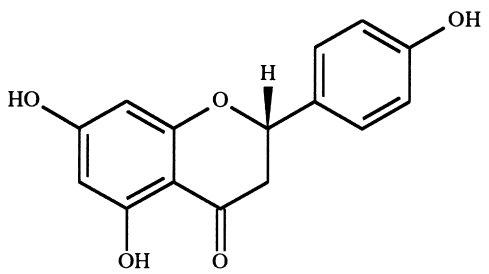
Abstract

An isocratic high-performance liquid chromatographic method with ultraviolet detection was utilized for the investigation of the pharmacokinetics of naringenin and its glucuronide conjugate in rat plasma and brain tissue. Plasma and brain tissue were deproteinized by acetonitrile, then centrifuged for sample clean-up. The drugs were separated by a reversed-phase C_{18} column with a mobile phase consisting of acetonitrile–orthophosphoric acid solution (pH 2.5–2.8) (36:64, v/v). The detection limits of naringenin in rat plasma and brain tissue were 50 ng/ml and 0.4 μg/g, respectively. The glucuronide conjugate of naringenin was evaluated by the deconjugated enzyme β-glucuronidase. The naringenin conjugation ratios in rat plasma and brain tissue were 0.86 and 0.22, respectively, 10 min after naringenin (20 mg/kg, i.v.) administration. The mean naringenin conjugation ratio in plasma was approximately four fold that in brain tissue.

Keywords: Naringenin

1. Introduction

Naringenin (Fig. 1) is thought to be one of the active components in grapefruit juice, which modulates the enzyme activity of cytochrome P-450. Concomitant intake of grapefruit juice alters the pharmacokinetics of a variety of drugs [1]. Citrus flavonoids exert various pharmacological activities, such as the inhibition of selected cytochrome P-450 isoform CYP1A2 [1,2], and CYP3A4 [3], which may increase oral bioavailability of nifedipine [4], cyclosporine [4], nimodipine, [5] and verapamil [6]. Little



naringenin

Fig. 1. Chemical structure of naringenin.

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is known about naringenin and its conjugation in the metabolic pathways of blood and brain.

Recently, several methods for the determination of naringenin have been described. They include HPLC–UV [7–9], HPLC with photodiode-array [10], gas chromatography [11], gas chromatography with mass spectrometry [12], liquid chromatography with mass spectrometry [10], and capillary electrophoresis [13]. However, most of the methods for naringenin measurement are not specific for the simultaneous determination of naringenin and its glucuronide conjugate. In this study, an isocratic HPLC method was used for the determination of naringenin and its glucuronide conjugate in rat plasma and brain tissue, after naringenin (20 mg/kg, i.v.) administration.

2. Experimental

2.1. Chemicals and reagents

Naringenin (Fig. 1), and β-glucuronidase (EC 3.2.1.31, type H-1, 330 000 units/g solid also contains sulfatase activity) from *Helix pomatia* were purchased from Sigma (St. Louis, MO, USA). Hydrocortisone acetate, as internal standard, was supplied by Nutritional Biochemical Corporation (Cleveland, OH, USA). All solvents were of analytical or HPLC grade and were obtained from BDH (Poole, UK). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations. Authentic naringenin (0.1, 1 and 10 μg/ml) and hydrocortisone acetate (80 μg/ml) in methanol and acetonitrile, respectively, were stored at 4°C.

2.2. Apparatus and chromatography

The HPLC system consisted of a Rheodyne injector (20-μl loop), a variable wavelength UV–Vis detector (Soma, Tokyo, Japan) set at 283 nm, and a chromatographic pump (Model PM-80, Bioanalytical System, West Lafayette, IN, USA). Separation was achieved on a reversed-phase Cosmosil (Nacalai Tesque, Tokyo, Japan) $5C_{18}$-AR column (250×4.6 mm I.D., particle size 5 μm) at room temperature (22–24°C). The mobile phase consisted of acetonitrile–orthophosphoric acid solution, pH 2.5–2.8 (36:64, v/v) at a flow-rate of 1.0 ml/min.

2.3. Animals

Male Sprague–Dawley rats (250–350 g) were obtained from the Laboratory Animal Center at National Yang-Ming University. These animals were specifically pathogen-free and kept in our own environmentally controlled quarters (at 24±1°C with 12–12 h light–dark cycle) for at least 1 week before use. Standard laboratory food and water were available at all times.

2.4. Blood sampling

Rats were anaesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). An additional dose of 10 mg/kg pentobarbital was given when the rat showed signs of awakening during the experiment. Blood samples (0.3 ml) were collected via cardiac puncture [14] at 5, 10, 20 and 30 min after naringenin (20 mg/kg, i.v.) administration.

2.5. Treatment of blood and brain tissue samples

Each collected blood sample was transferred to a heparinized microcentrifuge tube and centrifuged at 8000 *g* for 5 min (Eppendorf Model 5402, Hamburg, Germany). The resulting plasma (0.1 ml) was then mixed with 0.2 ml of acetonitrile containing hydrocortisone acetate as the internal standard. The denatured protein precipitate was separated by centrifugation at 8000 *g* for 5 min at 4°C, and a 20-μl aliquot of the supernatant was directly injected onto the HPLC system. The same sample handling process was used for recovery, precision and accuracy determinations.

In the brain tissue study, animals were killed by decapitation, and the various brain parts (cerebral cortex, striatum, cerebellum, hippocampus, brain stem, and rest of brain) were separated and weighed. The brain tissues were mixed with Ringer solution, 2 ml/g tissue, and homogenized in a laboratory homogenizer (Kinematica; Polytron, Littau-Luzern, Switzerland). The homogenate (0.05 ml) was then mixed with 0.5 ml of acetonitrile containing hydrocortisone acetate as the internal standard. The dena-

tured protein precipitate was separated by centrifugation at 8000 *g* for 5 min at 4°C, and a 20-μl aliquot of the supernatant was directly injected onto the HPLC system. The same sample handling process was used for recovery, precision and accuracy determinations of rat brain tissue [15].

2.6. Conjugated sample treatment

As described by Boutin et al. [16], incubation of β-glucuronidase and plasma or brain tissues was optimized in a 37°C water bath for 1 h in our preliminary studies of naringenin deconjugation. For naringenin deconjugation, aliquots (250 μl) of plasma or brain tissue homogenate were incubated with 50 μl β-glucuronidase in a 37°C water bath for 1 h. Following incubation, 0.1 ml incubated plasma or 0.05 ml brain tissue homogenate were mixed with 0.2 ml or 0.5 ml of acetonitrile containing internal standard, to denature the proteins.

2.7. Recovery

Absolute recovery has been defined as a measure of the efficiency of the extraction of the analyte from the sample matrix [17]. In the experiment, recoveries of naringenin were determined at low, moderate and high concentrations of 2, 10 and 50 μg/ml from plasma samples, and 4, 20 and 100 μg/g from brain tissues, respectively.

$$\text{Absolute recovery} = 100 \times \frac{\text{response of analyte spiked into matrix}}{\text{response of analyte of pure standard}}$$

2.8. Precision and accuracy

The precision over the entire working concentration range was determined by replicate analyses of rat plasma samples ($n=6$) or brain tissue homogenates ($n=6$) spiked with three concentrations of naringenin. Precision was expressed as the percentage relative standard deviation (R.S.D.) of the replicate measurements. To determine the intra-assay variation, replicate assays were carried out on the analytical system at different times during the day. The inter-assay variation was determined by assaying the spiked samples on days 1, 2, 4 and 6. Relative standard deviations were calculated from the following equation [17,18]:

$$\%\text{R.S.D.} = 100 \times \left(\frac{\text{standard deviation}}{\text{mean}}\right)$$

The accuracy was defined as the agreement between the measured value and the true value. The percentage bias was calculated from the expression [17]:

$$\%\text{bias} = 100 \times \frac{\text{measured value} - \text{true value}}{\text{true value}}$$

3. Results and discussion

The retention times of naringenin and hydrocortisone acetate (internal standard) in rat plasma and brain tissue were found to be 8.0 and 11.5 min, respectively (Figs. 2 and 3). The purity of naringenin

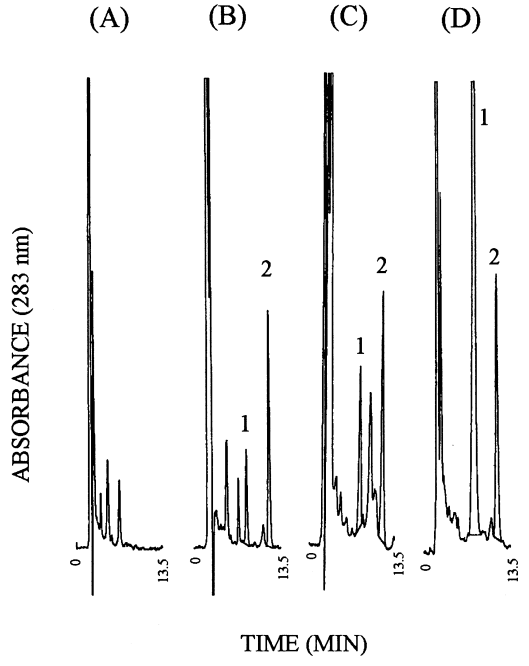

Fig. 2. Chromatograms of naringenin in rat plasma: (A) blank plasma, (B) plasma spiked with naringenin (0.2 μg/ml) and hydrocortisone acetate (internal standard), (C) plasma sample 10 min after 20 mg/kg intravenous administration of naringenin, (D) 1-h incubation with β-glucuronidase and (C) sample. 1= naringenin; 2=hydrocortisone acetate (internal standard).

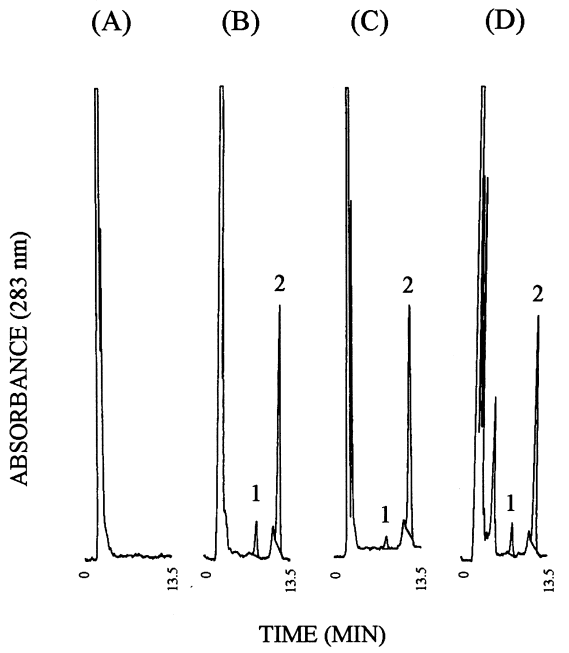

Fig. 3. Chromatograms of naringenin in rat brain tissue: (A) blank brain tissue, (B) brain tissue spiked with naringenin (1 μg/g) and hydrocortisone acetate (internal standard), (C) brain tissue sample 10 min after 20 mg/kg intravenous administration of naringenin, (D) 1-h incubation with β-glucuronidase and (C) sample. 1= naringenin; 2=hydrocortisone acetate (internal standard).

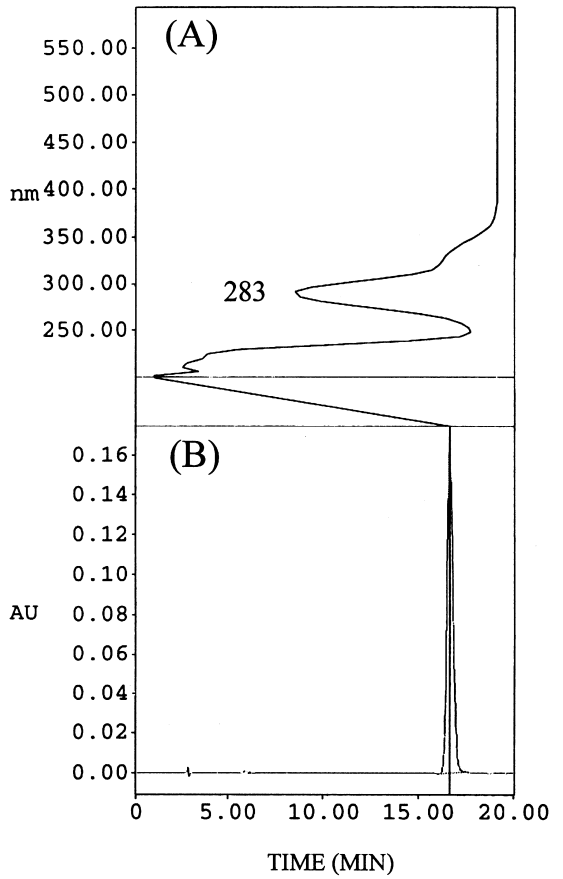

Fig. 4. UV spectrum (A) and chromatogram (B) of authentic naringenin, measured by HPLC coupled with photodiode-array detection (Waters, Model 996, Milford, MA, USA) for purity test and identification.

and the maximum UV absorption at 283 nm were measured by photodiode-array (Fig. 4). The complementary use of the photodiode-array for identifying the analyzed compound by wavelength absorption was more reliable than UV detection alone. The pH value and solvent system were important factors for improving the separation of naringenin in biological samples. The optimum pH range of orthophosphoric acid solution was 2.5–2.8. Not only orthophosphoric acid, but also acetic and formic acids improved the separation of the naringenin sample [8]. The use of acetonitrile instead of methanol improved the separation and shortened the retention times of analytes.

Fig. 2A shows the chromatogram of blank rat plasma. No discernible peaks were observed within the time frame in which naringenin and internal standard were detected. Fig. 2B shows the chromatogram of rat plasma spiked with naringenin (0.2 μg/ml) and internal standard (hydrocortisone). Fig. 2C shows the chromatogram of naringenin plasma sample obtained 10 min after naringenin (20 mg/kg, i.v.) administration to a rat. Fig. 2D shows the chromatogram of naringenin plasma sample obtained as in Fig. 2C, and then incubated with β-glucuronidase in water bath (37°C) for 1 h.

Fig. 3A shows the chromatogram of blank homogenate of rat brain tissue. No discernible peaks were observed within the time frame in which naringenin and internal standard were detected. Fig.

3B shows the chromatogram of rat brain tissue homogenate spiked with naringenin (2 μg/ml) and internal standard (hydrocortisone). Fig. 3C shows the chromatogram of naringenin brain tissue homogenate sample obtained 10 min after naringenin (20 mg/kg, i.v.) administration to a rat. Fig. 3D shows the chromatogram of naringenin sample from brain tissue homogenization obtained as in Fig. 3C and then incubated with β-glucuronidase in water bath (37°C) for 1 h.

To determine the linearity and the detection limit of the HPLC method, rat plasma and brain tissue samples were analyzed and spiked with naringenin concentrations of 0.1–50 μg/ml and 0.5–100 μg/g, respectively. The peak area ratios (naringenin to hydrocortisone) were linearly related to the concentration of naringenin (correlation coefficient, $r^2 = 0.999$) and the equations for the regression lines for naringenin in rat plasma and brain tissue homogenate were found to be $y = 0.61x + 0.047$ and $y = 15.5x + 0.059$, respectively. The detection limits for naringenin, at a signal-to-noise ratio of 3:1, were found to be 0.05 μg/ml and 0.4 μg/g for rat plasma and brain tissue homogenate, respectively.

The recoveries of naringenin from rat plasma were 103.7, 107.9, and 102.8% for concentrations of 2, 10 and 50 μg/ml, respectively. The recoveries of naringenin from rat brain tissue homogenate were 88.3, 96.8 and 100.0% for concentrations of 4, 20 and 100 μg/g, respectively.

The reproducibility of the method was defined by both intra-assay and inter-assay variabilities. The intra-assay and inter-assay R.S.D.s for naringenin in rat plasma and brain tissue were within 15% which is acceptable [17] (Tables 1 and 2). Accuracy of a bioanalytical method is a measure of the systematic error or bias. The intra-assay and inter-assay biases of the naringenin samples were less than 10% [17] (Tables 1 and 2).

The plasma and cerebral cortex concentrations of total form (conjugation and free) and free form naringenin were 4.84±0.30 and 0.68±0.09 μg/ml, and 2.11±0.4 and 1.65±0.19 μg/g, respectively, 10 min after naringenin administration (20 mg/kg, i.v.). In addition, the conjugated ratios (free/total of naringenin) in rat plasma and brain were 0.86 and 0.22, respectively. The results suggest that the concentration of free form naringenin in brain is higher than in peripheral plasma. However, the concentration of total naringenin in brain is less than in plasma.

Table 1
Validation of assay for naringenin in rat plasma ($n = 6$ for all measurements)

Concentration (μg/ml)		R.S.D. (%)	Bias (%)
Added	Found		
Intra-assay			
2	2.0±0.1	5.8	−1.1
10	10.0±0.2	2.3	−0.1
50	50.0±0.04	0.08	−0.003
Inter-assay			
2	1.9±0.2	10.2	−5.7
10	10.2±0.5	4.9	1.9
50	50.0±0.09	0.2	−0.1

Data are expressed as mean±S.D.

Recently, determinations of naringin and naringenin in human plasma have been published [8]. However, to develop a chromatographic method for the measurement of naringenin in biological sample, the glucuronidation form must be considered, with attention to avoiding the interference of conjugated peak on the chromatogram. Because naringenin has potential therapeutic interaction properties through the inhibition of cytochrome P-450, grapefruit juice

Table 2
Validation of assay for naringenin in rat brain tissue ($n = 6$ for all measurements)

Concentration (μg/ml)		R.S.D. (%)	Bias (%)
Added	Found		
Intra-assay			
1	1.0±0.03	3.1	−1.8
4	4.0±0.1	3.5	−0.7
20	20.0±0.09	0.4	−0.3
Inter-assay			
1	0.9±0.04	4.9	−8.1
4	4.1±0.5	4.9	1.5
20	20.0±0.2	0.8	−0.2

Data are expressed as mean±S.D.

is now known to enhance the therapeutic or toxic effects of several clinically important drugs [19], flavonoids and nonflavonoid compound [20]. These drugs interactions affect both peripheral and central nervous systems.

4. Conclusions

This isocratic HPLC method has been successfully applied to the determinations of naringenin in plasma and brain tissue using the same chromatographic system. Its value for in vivo monitoring of rat plasma and brain tissue levels has been demonstrated. This method is therefore suitable for tissue distribution, bioavailability and pharmacokinetic studies, as well as clinical drug interaction monitoring. Conceivably, this method could also be adapted for the analyses of similar compounds, which exhibit glucuronidation and brain tissue distribution.

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