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Determination of naringin and naringenin in human plasma by high-performance liquid chromatography

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Abstract

An HPLC method for determining a flavonoid, naringin, and its metabolite, naringenin, in human plasma is presented for application to the pharmacokinetic study of naringin. Isocratic reversed-phase HPLC was employed for the quantitative analysis by using genistin (for naringin) or daidzein (for naringenin) as an internal standard and solid-phase extraction using a Sep-Pak t C_{18} cartridge. For the determination, HPLC was carried out using an Inertsil ODS-2 column (250×4.6 m I.D., 5 μm particle size). The mobile phases were acetonitrile–0.1 *M* ammonium acetate solution (20:80, v/v; pH 7.1) for naringin and acetonitrile–0.1 *M* ammonium acetate solution–acetic acid (30:69:1, v/v; pH 4.9) for naringenin. The flow-rate was 1 ml min^{-1}. The analyses were performed by monitoring the wavelength of maximum UV absorbance at 280 nm for naringin and at 292 nm for naringenin. The detection limits on-column were about 0.2 ng for the two flavonoids.

Keywords: Naringin; Naringenin; Flavonoids

1. Introduction

Flavonoids are a group of naturally occurring compounds ubiquitous in the plant kingdom. These flavonoids have shown many biological and pharmacological activities, such as the inhibition of enzymes [1,2], free radical scavenging [3], anti-inflammation [4], anti-estrogen [5] and the inhibition of tumor promotion [6].

A flavonoid glycoside naringin, a "bitter principal", 4′,5,7-trihydroxyflavanone-7-rhamnoglucoside, is a major flavonoid present in grapefruits and constitutes up to 10% of the dry weight [7]. Recent in vivo studies in humans have shown dramatic effects of grapefruit juice on the oxidation of dihydropyridine calcium channel blockers [8,9] and on lowering elevated hematocrits in human subjects [10]. The grapefruit juice and naringenin (the aglycone from naringin) have also been demonstrated to inhibit the human cytochrome P-450 isoform, CYP1A2, in vivo [11]. In vitro naringenin inhibits CYP3A4 activity in human liver microsomes [12].

Naringin (Fig. 1) and several other flavonoids which possess a glucoside moiety at the 7-position of the flavonoid skeleton, such as rhoifolin (4′,5,7-trihydroxyflavone-7-rhamnoglucoside) and daidzein (4′,7-dihydroxy-isoflavone-7-glucoside) also have the ability to activate polymorphonuclear leukocyte (PMN) to induce the cytotoxic activity of PMN against tumor cells in vitro [13]. The position and identity of the sugar moiety have been shown to be of significant importance in activating PMN.

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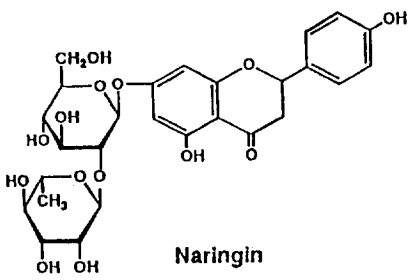

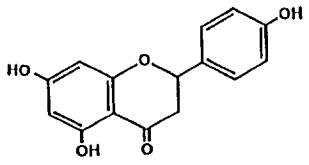

Fig. 1. Structures of naringin and naringenin.

In an attempt to conduct pharmacokinetic studies of rhoifolin and daidzein, we have already described methods for their determination in human plasma by HPLC [14]. This paper deals with the quantitative determination of naringin and naringenin in human plasma by HPLC.

2. Experimental

2.1. Chemicals and reagents

Naringin was purchased from Fluka (Buchs, Switzerland). Naringenin, genistin and daidzein were purchased from Extrasyntase (Genay, France). Naringenin was purified by silica gel column chromatography using ether–benzene (1:1, v/v), followed by recrystallization from aqueous ethanol before use. Stock solutions of naringenin were prepared by dissolving in ethanol. Stock solutions of naringin, genistin and daidzein were prepared by dissolving in methanol. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Sample preparation

To 1.0-ml aliquots of human plasma were added different amounts of naringin (16.9–202.6 ng) and a fixed amount (103.5 ng) of genistin as an internal standard. For the determination of naringenin, different amounts of naringenin (33.4–167.8 ng) and a fixed amount (101.6 ng) of daidzein (as an internal standard) were used. The plasma sample was diluted with 2 ml of water and applied to a Sep-Pak t C_{18} cartridge (Waters, Milford, MA, USA), which had previously been conditioned by washing with 5 ml of methanol and 5 ml of distilled water. The cartridge was first washed with 2.5 ml of distilled water, purged with air and then eluted with 5 ml of 80% methanol. After evaporating the eluate at room temperature in vacuo, the residue was dissolved in 2 ml of methanol for naringin or 2 ml of ethanol for naringenin and the solution was filtered through an HLC-Disc filter (Kanto Chemicals, Tokyo, Japan). The filtrate was transferred to a spitz tube with a ground-glass joint and evaporated to dryness at room temperature in vacuo. The residue was dissolved in 100 μl of the mobile phase with vortex-mixing for 30 s for the naringin analysis. For naringenin, the residue was dissolved in 50 μl of ethanol with vortex-mixing for 30 s and then 50 μl of the mobile phase. A 30-μl portion of the solution was subjected to HPLC in duplicate.

2.3. Apparatus and HPLC conditions

HPLC analyses were performed on a Jasco (Tokyo, Japan) liquid chromatograph equipped with a Model 880 PU pump and a Model 875 UV–Vis detector. Data processing was performed with a Model 12 Sic chromatocorder (System Instrument, Tokyo, Japan). The 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. For the analysis of naringin, the mobile phase was acetonitrile–0.1 *M* ammonium acetate solution (20:80, v/v; pH 7.1) and the flow-rate was 1.0 ml min^{-1}. The detection wavelength was set to 280 nm. For the analysis of naringenin, the mobile phase was acetonitrile–0.1 *M* ammonium acetate solution–glacial acetic acid (30:69:1, v/v; pH 4.9) and the

flow-rate was 1.0 ml min^{-1}. Naringenin was detected at 292 nm, which is the wavelength of maximum absorbance of naringenin in the mobile phase.

2.4. Calibration

To each standard containing known amounts of naringin (16.9, 50.7, 101.3 and 202.6 ng) or naringenin (33.4, 66.7, 100.1, 133.4 and 167.8 ng) were added 103.5 ng of genistin (for naringin) or 101.6 ng of daidzein (for naringenin) as the internal standard. Each sample was prepared in duplicate. After evaporating the solvent to dryness, the residue was dissolved in 100 μl of the mobile phase for naringin or in 100 μl of ethanol–mobile phase (50:50, v/v) for naringenin. A 30-μl portion of the solution was subjected to HPLC. The peak-area ratios (naringin to genistin, or naringenin to daidzein) were determined in duplicate. The calibration graphs were obtained by a least-squares linear fitting of the peak-area ratios versus the amounts of genistin or daidzein.

2.5. Accuracy

Accuracy was determined for naringin and naringenin by assaying six preparations of 1.0-ml aliquots of human plasma containing two different amounts of naringin (33.7 and 140.6 ng) or naringenin (50.1 and 150.3 ng) and a fixed amount of genistin (103.5 ng) or daidzein (101.6 ng) as the internal standard. After preparing the sample for HPLC as described above, the peak-area ratios (naringin or naringenin to the respective internal standard) were determined.

2.6. Sensitivity

Sensitivity was determined by injecting standard solutions containing known amounts (0.5–0.2 ng) of naringin or naringenin into the isocratic reversed-phase HPLC system, with UV detection at 280 nm for naringin or at 292 nm for naringenin.

3. Results and discussion

Reversed-phase HPLC with the ODS column is often used for the purification and separation of naturally occurring flavonoids in crude plant materials and food products (aglycones and glycosides). The method provides good separation, high resolution and sensitivity. The acetonitrile–water system as well as the methanol–water system are generally used as the mobile phase. The addition of acetic- or formic acid to their solvent systems improves the separation, but the systems without the acid were also successful in a number of studies, as shown in the case of isoflavonoids [15–17].

For further determination of flavonoids by the liquid chromatography–thermospray mass spectrometry method, the isocratic RP-HPLC was performed with the eluents containing ammonium acetate. The HPLC behaviour of naringin and its aglycone, naringenin, were first examined by using an Inertsil ODS-2 reversed-phase column as described in our previous report [14]. The solvent system consisting of acetonitrile and 0.1 *M* ammonium acetate (20:80, v/v; pH 7.1) was used for the determination of naringin. The mobile phase containing methanol was inappropriate because it produced very broad peaks. The acetonitrile–0.1 *M* ammonium acetate system resulted in peak tailing, on analysis of naringenin. The addition of acetic acid (1%, v/v) to the solvent system, however, improved the separation to a large extent.

For the successful determination of naringin and naringenin in human plasma by HPLC, it is essential that the analytes are well recovered from plasma and are separated on the chromatogram from interfering material present in the plasma. Sep-Pak C_{18} cartridges provide a simple method for the extraction and clean-up of flavonoids from plant extracts [18,19], food material [20] and from biological fluids [21–23] prior to HPLC or GC analysis. To efficiently extract naringin and naringenin from human plasma, the use of Sep-Pak C_{18} seemed promising, as employed in our previous study on the analysis of two flavonoid glycosides, daidzein and rhoifolin. When 80% aqueous methanol was used as the eluting solvent for the Sep-Pak C_{18} cartridge, it was found that good recoveries of naringin and naringenin were obtained and that these were well resolved from the interfering peaks on the HPLC chromatogram. Elution with 80% aqueous methanol gave recoveries of 93.2–104.8% ($n=4$) for naringin and 97.8–103.5% ($n=2$) for naringenin. Fig. 2A shows a typical HPLC profile of the HPLC chromatogram of

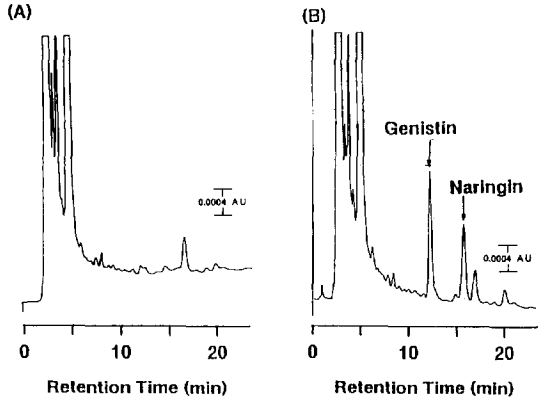

Fig. 2. HPLC of extracts of (A) blank plasma and (B) plasma spiked with naringin (126.5 ng) and genistin (103.5 ng).

human plasma without spiking naringin and the internal standard. Fig. 2B illustrates the chromatogram of an extract of human plasma spiked with naringin (126.5 ng) and genistin (103.5 ng). Comparison of the chromatograms shown in Fig. 2A and 2B demonstrates that there is no significant interference from endogenous components for the analysis of naringin. Fig. 3A and 3B illustrate the chromatograms of extracts of human plasma (Fig. 3A) and human plasma spiked with naringenin (150.3 ng) and with the internal standard, daidzein (101.6 ng) (Fig. 3B). In Fig. 3A, a small peak was observed at the retention time of naringenin. Presently, it has not been confirmed whether this peak results from the endogenous naringenin or not. The peak, however, was found to correspond to 10 ng/ml of naringenin and the interference was not significant when determining human plasma naringenin concentrations higher than 30 ng/ml.

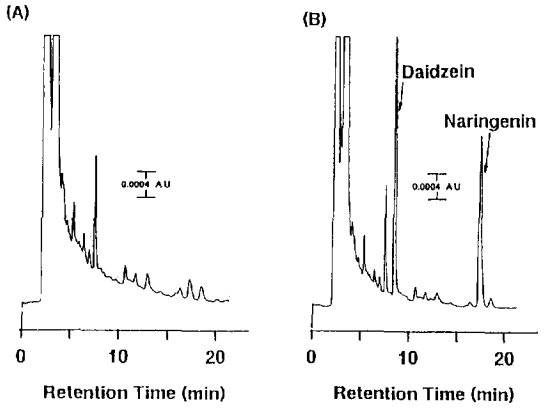

Fig. 3. HPLC of extracts of (A) blank plasma and (B) plasma spiked with naringenin (150.3 ng) and daidzein (101.6 ng).

Calibration graphs were prepared in the ranges 20–200 ng of naringin and 30–170 ng of naringenin, using genistin or daidzein as the internal standard. The peak-area ratios were plotted against the mixed mass ratios of naringin or naringenin to the respective internal standard. A good correlation was found between the observed peak-area ratios (y) and the mixed mass ratios (x). A least-squares regression analysis gave the regression lines $y=0.0068x+0.0955$ ($r=0.999$) for naringin and $y=0.0053x+0.0418$ ($r=0.996$) for naringenin.

The accuracy of measurements was determined by adding known amounts of naringin or naringenin to 1.0 ml aliquots of pooled plasma. To the plasma samples were added fixed amounts of genistin (103.5 ng) or daidzein (101.6 ng) and different amounts of naringin (33.7 and 140.6 ng) or naringenin (50.1 and 150.3 ng). Table 1 shows that the amounts of naringin or naringenin added were in good agreement with the amounts of naringin or naringenin measured, the relative errors being less than 8% for

Table 1
Accuracy of HPLC determination of naringin and naringenin in plasma

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values						Mean±S.D.		
Naringin									
33.7	37.9	35.9	37.3	37.8	33.6	36.0	36.4±1.63	+8.0	4.5
140.6	144.6	146.3	147.3	138.1	139.1	148.1	143.9±4.29	+2.3	3.1
Naringenin									
50.1	52.8	51.8	48.0	52.2	57.3	54.7	52.7±3.14	+5.2	6.0
150.3	151.9	156.6	156.0	159.5	156.3	148.1	154.7±4.06	+2.8	2.6

naringin and 6% for naringenin. The inter-assay relative standard deviations (R.S.D.s) ($n=6$) were less than 6% for both naringin and naringenin.

The sensitivity of the HPLC assay was 0.2 ng for naringin and 0.25 ng for naringenin per injection, with a signal-to-noise ratio of about 4.

The present method provides a sensitive and reliable technique for the determination of plasma concentrations of naringin and naringenin. Using genistin or daidzein as the internal standard for the reversed-phase HPLC assay, good accuracy and precision are obtained.

References

[1] B. Havesteen, Biochem. Pharmacol., 32 (1983) 1141.
[2] W.-M. Keung and B.L. Vallee, Proc. Natl. Acad. Sci. U.S.A., 90 (1993) 1247.
[3] W. Bors, W. Heller, C. Michel and M. Saran, Methods Enzymol., 186 (1990) 343.
[4] T. Di Perri and A. Auteri, Int. Angiol., 7 (1988) 11.
[5] B.Y. Tang and N.R. Adams, J. Endocrinol., 85 (1980) 291.
[6] H. Nishino, M. Nagao, H. Fujiki and T. Sugimura, Cancer Lett., 21 (1983) 1.
[7] R.F. Albach and G.H. Reolman, Phytochemistry, 8 (1969) 127.
[8] D.G. Bailey, B. Edgar, J.D. Spence, C. Munoz and J.M.O. Arnold, Clin. Pharmacol. Ther., 47 (1990) 180.
[9] B. Edgar, D.G. Bailey, R. Bergstrand, G. Johnsson and L. Lurje, Clin. Pharmacol. Ther., 47 (1990) 181.
[10] R.C. Robbins, F.G. Martin and J.M. Roe, Int. J. Vitam. Nutr. Res., 58 (1988) 414.
[11] U. Fuhr, K. Klittich and A.H. Staib, Br. J. Clin. Pharmacol., 35 (1993) 431.
[12] F.P. Guengerich and D.H. Kim, Carcinogenesis, 11 (1990) 2275.
[13] K. Morikawa, Cell Sci., 6 (1990) 532.
[14] K. Ishii, S. Sumiko, T. Furuta and Y. Kasuya, J. Chromatogr. B, 665 (1994) 300.
[15] P.A. Murphy, J. Chromatogr., 241 (1981) 166.
[16] E. Farmakalidis and P.A. Murphy, J. Chromatogr., 295 (1984) 510.
[17] E. Farmakalidis and P.A. Murphy, J. Agric. Food Chem., 33 (1985) 385.
[18] P. Pietta, A. Bruno, P. Mauri and A. Rava, J. Chromatogr., 593 (1992) 165.
[19] M. De Bernardi, E. Uberti, G. Vidari and O. Servettaz, J. Chromatogr., 284 (1984) 269.
[20] A. Seo and C.V. Morr, J. Agric. Food Chem., 32 (1984) 530.
[21] C. Bannwart, T. Fotsis, R. Heikkinen and H. Adlercreutz, Clin. Chim. Acta, 136 (1984) 165.
[22] P.E. Juniewicz, S.P. Morell, A. Moser and L.L. Ewing, J. Steroid Biochem., 31 (1988) 987.
[23] T.J.-O. Lundh, H.I. Pettersson and K.A. Martinsson, J. Agric. Food Chem., 38 (1990) 1530.