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Journal of Chromatography B, 704 (1997) 299–305

JOURNAL OF CHROMATOGRAPHY B

Determination of naringin and naringenin in human urine by high-performance liquid chromatography utilizing solid-phase extraction

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Received 29 April 1997; received in revised form 22 September 1997; accepted 22 September 1997

Abstract

An HPLC method for determining a flavonoid naringin and its metabolite, naringenin, in human urine is presented for application to the pharmacokinetic study of naringin. Isocratic reversed-phase HPLC was employed for the quantitative analysis by using hesperidin for naringin or hesperetin for naringenin as internal standard and solid-phase extraction using a strong anion exchanger, Sep-Pak Accell QMA cartridge. The HPLC assay was carried out using an Inertsil ODS-2 column (250×4.6 mm I.D., 5 μm particle size). The mobile phases were acetonitrile–0.1 *M* ammonium acetate–acetic acid (18:81:1, v/v; pH 4.7) for naringin and acetonitrile–0.1 *M* ammonium acetate–triethylamine (25:75:0.05; v/v; pH 8.0) for naringenin. The flow-rate was 1.0 ml min^{-1}. The analyses were performed by monitoring the wavelength of maximum UV absorbance at 282 nm for naringin and at 324 nm for naringenin. The lower limits of quantification were ca. 25 ng/ml for naringin and naringenin with R.S.D. less than 10%. The lower limits of detection (defined as a signal-to-noise ratio of about 3) were approximately 5 ng for naringin and 1 ng for naringenin. A preliminary experiment to investigate the urinary excretion of naringin, naringenin and naringenin glucuronides after oral administration of 500 mg of naringin to a healthy volunteer demonstrated that the present method was suitable for determining naringin and naringenin in human urine.

Keywords: Naringin; Naringenin

1. Introduction

A flavonoid glycoside naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) (Fig. 1) is a major flavonoid present in grapefruits [1]. Naringin and several other flavonoids which possess a glycoside moiety at the 7-position of the flavonoid skeleton, such as rhoifolin (4′,5,7-trihydroxyflavone-7-rhamnoglucoside) and daidzin (4′,7-dihydroxyisoflavone-7-glucoside), have the ability to activate polymorphonuclear leucocytes (PMN) against tumor cells in vitro [2]. The position and identity of the sugar moiety have been shown to be of significant impor-

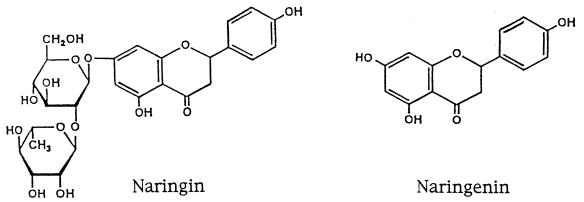

Fig. 1. Structures of naringin and naringenin.

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0378-4347/97/$17.00
PII S0378-4347(97)00474-X

tance in activating PMN. Recent studies in humans have shown biological activities of grapefruit juice, such as the oxidative enzyme inhibition [3,4] and the lowering effect on the elevated hematocrits [5].

In order to obtain detailed pharmacokinetic information and establish the bioavailability of naringin after oral administration, both plasma and urine data are required. After administration of grapefruit juice, the evaluation of renal excretion of naringin, and naringenin (Fig. 1) and naringenin glucuronides were reported [6]. The disposition of citrus flavonoids (naringin and hesperidin) was also evaluated after single oral doses [7]. However, the determination of naringin in human urine has not been reported. In an attempt to characterize pharmacokinetic properties of flavonoids, we have already described assay methods for determining rhoifolin and daidzin [8] and naringin [9] in human plasma using HPLC without hydrolysis of sugar moiety. This paper deals with the quantitative determination of naringin and its metabolite, naringenin, in human urine by HPLC. The application data of this HPLC method were also presented.

2. Experimental

2.1. Chemicals and reagents

Naringin, naringenin, hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) and hesperidin (hesperetin-7-rhamnoglucoside) were purchased from Extrasyntase (HPLC grade, Genay, France). Stock solutions of naringin and hesperidin were prepared by dissolving in methanol. Stock solutions of naringenin and hesperetin were prepared by dissolving in ethanol. 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 urine were added different amounts of naringin (23.8–213.8 ng) and a fixed amount (166.3 ng) of hesperidin as internal standard. For the determination of naringenin, different amounts of naringenin (22.5–157.5 ng) and a fixed amount of hesperetin (130.0 ng) as internal standard were used. For naringin, the urine sample was diluted with 2 ml of water and applied to a strong anion exchanger, Sep-Pak Accell QMA cartridge (2 g packing, Waters, Milford, MA, USA), which had previously been conditioned by washing with 15 ml of methanol and 15 ml of distilled water. The cartridge was first washed with 1.0 ml of distilled water. After purging with air, the cartridge was eluted with 10.0 ml of 1 m*M* formic acid in methanol. The flow-rate of elution from the cartridge was 3 ml min^{-1}. For naringenin, the urine sample was diluted with 2 ml of water and applied to a Sep-Pak Accell Plus QMA cartridge (360 mg packing), 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. After purging with air, the cartridge was eluted with 7.0 ml of 4 m*M* formic acid in methanol. The flow-rate of elution from the cartridge was 3 ml min^{-1}. 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 with vortex-mixing for 30 s. A 40 μl portion of the solution was subjected to HPLC.

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 detector. The mobile phase was degassed with a Model DG-980-51 degasser. Data processing was carried out with a Model 12 Sic chromatocorder (System Instrument, Tokyo, Japan). The HPLC system was 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–

glacial acetic acid (18:81:1, v/v; pH 4.7) and the flow-rate was 1.0 ml min^{-1}. The detection wavelength was set at 282 nm. For the analysis of naringenin, the mobile phase was acetonitrile–0.1 *M* ammonium acetate–triethylamine (25:75:0.05, v/v; pH 8.0) and the flow-rate was 1.0 ml min^{-1}. Naringenin was detected at 324 nm, the wavelength of maximum absorbance of naringenin in the mobile phase.

2.4. Recovery

The absolute recovery was assessed at two concentration levels of 142.5 ng/ml and 1068.8 ng/ml in urine for naringin or 90.0 ng/ml and 1080.0 ng/ml in urine for naringenin. The urine samples were applied to a Sep-Pak Accell QMA cartridge as described above. The absolute recoveries for naringin and naringenin were calculated by comparing the peak-height ratios of the HPLC chromatograms before and after the extraction procedure.

2.5. Calibration

Standard samples were prepared by adding known amounts of naringin (35.6, 71.3, 118.8, 166.3, 213.8 ng) or naringenin (22.5, 67.5, 112.5, 157.5 ng) to blank urine. To each standard were then added 166.3 ng of hesperidin (for naringin) or 130.0 ng of hesperetin (for naringenin) as the internal standard. The standard samples were prepared in duplicate and triplicate measurements were made for each sample. Standard curves were generated following the extraction and HPLC analyses of the spiked urine samples. After determining the peak-height ratios (naringin to hesperidin, or naringenin to hesperetin) of the HPLC chromatograms, the calibration graphs were obtained by a least-squares linear fitting of the peak-height ratios vs. the amounts of hesperidin or hesperetin.

2.6. Accuracy

Accuracy was determined for naringin and naringenin by assaying six preparations of 1.0 ml aliquots of human urine containing three different amounts of naringin (23.8, 47.5 and 142.5 ng) or naringenin (22.5, 45.0 and 135.0 ng) and a fixed amount of hesperidin (166.3 ng) or hesperetin (130.0 ng) as the internal standard. After preparing the samples for HPLC as described above, the peak-height ratios (naringin or naringenin to the respective internal standard) were determined.

2.7. Stability in urine

Stability of naringin and naringenin in urine was examined at 37°C and −20°C. To the urine samples was added naringin (166.3 ng/ml) or naringenin (112.5 ng/ml) and the samples were incubated at 37°C for 5, 10, 24 h. Storage stability at −20°C was determined after storing the samples over a period of 90 days.

2.8. Application of the assay and sample collection

After a single oral administration of 500 mg of naringin to a 24 year-old male healthy volunteer, urine samples were collected just before administration and 0–2, 2–4, 4–8, 8–12, 12–16 and 16–24 h after administration. A 1.0 ml portion of each urine sample was directly used to determine naringin and naringenin and the urine sample was diluted four times on determining naringenin glucuronides. Naringenin glucuronides were determined as naringenin after the hydrolysis with β-glucuronidase according to the method of Axelson et al. [10].

3. Results and discussion

Reversed-phase HPLC has been widely utilized for the purification and separation of naturally occurring flavonoids (aglycones and glycosides) in crude plant materials and food products [11]. In an attempt to conduct pharmacokinetic studies of three flavonoid glycosides, naringin, rhoifolin and daidzin, we have already described methods for the determination of these glycosides in human plasma by reversed-phase HPLC using the ODS column [8,9]. A Sep-Pak C_{18} cartridge was successfully applied to the extraction and clean-up of these flavonoids from human plasma prior to the HPLC analysis.

Both plasma and urine data are often required to obtain detailed pharmacokinetic information. With an interest in characterizing the disposition and bio-

availability of naringin, an HPLC method for assaying naringin and its aglycone, naringenin, in human urine was developed in the present study. A simple extraction method using a Sep-Pak C_{18} cartridge was found not to effectively remove endogenous components in urine that severely interfered with the HPLC analysis. This caused difficulties in determining urinary naringin or naringenin by HPLC.

A combined use of Sep-Pak C_{18} cartridge, a cation exchanger and a strong anion exchanger is often a method of choice for the extraction of flavonoids in urine [12–16]. Combination of a Sep-Pak C_{18} cartridge and a Sep-Pak Accell QMA cartridge (2 g package for naringin or 360 mg package for naringenin) was found to effectively extract naringin and naringenin from urine and eliminate the interfering material. However, even the use of a Sep-Pak Accell QMA cartridge alone was found to result in the effective extraction and clean-up. There was no large difference in the chromatographic profiles between the two methods. The method using a Sep-Pak Accell QMA cartridge alone was then considered suitable for the routine analysis of naringin and naringenin in urine.

A solution of formic acid in methanol was used as the eluting solvent for the Sep-Pak Accell QMA cartridge to obtain good recoveries of naringin and naringenin and eliminate the interfering peaks on the HPLC chromatogram. The recoveries of naringin and naringenin from human urine were calculated by comparing the peak heights before and after the Sep-Pak Accell QMA extraction procedure. Elution with 1 m*M* formic acid in methanol gave recoveries of 94.4±3.5% (142.5 ng, $n=6$) and 92.3±3.2% (1068.8 ng, $n=3$) for naringin. Elution with 4 m*M* formic acid in methanol gave recoveries of 81.3±5.9% (90.0 ng, $n=6$) and 80.0±2.0%(1080.0 ng, $n=3$) for naringenin. Calibration of the overall analytical procedure gave a linear signal ($r>0.999$) up to approximately 1000 ng of both compounds in urine.

The HPLC behaviour of naringin and naringenin extracted from human urine was examined by using an Inertsil ODS-2 reversed-phase column. For the future analysis of flavonoids by liquid chromatography-thermospray mass spectrometry, the isocratic reversed-phase HPLC was performed with the eluents containing ammonium acetate. It has been demonstrated that the use of a solvent system consisted of acetonitrile–0.1 *M* ammonium acetate–acetic acid (18:81:1, v/v, pH 4.7) results in good separation of naringin and hesperidin in citrus juices by reversed-phase HPLC [17]. In the present study, then, this solvent system was chosen for the determination of naringin in urine. Fig. 2A shows a typical HPLC profile of the HPLC chromatogram of human urine without spiking naringin and the internal standard. Fig. 2B illustrates the chromatogram of an extract of human urine spiked with naringin (142.5 ng) and hesperidin (166.3 ng). Comparison of the chromatograms shown in Fig. 2A and Fig. 2B demonstrates that there is no significant interference from endogenous components for the analysis of naringin. Without adding acetic acid to the solvent system, a small peak appearing after naringin on the chromatogram overlapped with the naringin peak. Addition of acetic acid to the solvent system was, therefore, necessary to separate naringin from this interfering material present in urine.

The solvent system consisted of acetonitrile–0.1 *M* ammonium acetate (32:68, v/v, pH 7.4) resolved the naringenin peak from two large interfering peaks on the HPLC chromatogram. However, a difficulty was encountered on a choice of appropriate internal standard. That is, among possible candidates for the internal standard, daidzein overlapped with one interfering peak and hesperetin, genistein and apigenin overlapped with the other interfering peak. Using an acidic solvent system of acetonitrile–0.1 *M*

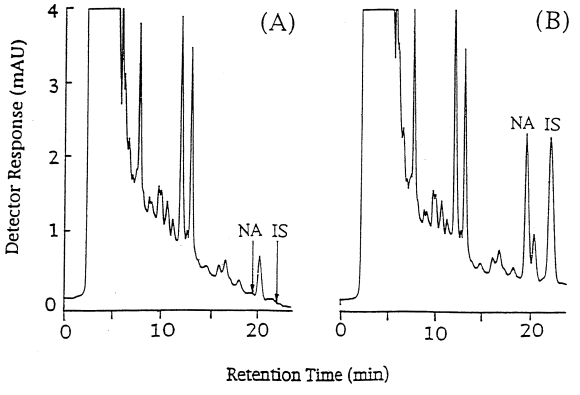

Fig. 2. HPLC chromatograms of extracts of (A) blank urine, and (B) urine spiked with naringin (142.5 ng/ml urine) and hesperidin (166.3 ng/ml urine) analyzed as described in the text. Peaks: NA=naringin; IS=hesperidin.

ammonium acetate–acetic acid (69:30:1,v/v, pH 5.0), naringenin was not resolved from the former interfering peak.

Successful results were obtained when a solvent system composed of acetonitrile–0.1 *M* ammonium acetate–triethylamine (25:75:0.05, v/v, pH 8.0) was used. Fig. 3 illustrates the chromatograms of extracts of human urine (Fig. 3A) and human urine spiked with 112.5 ng of naringenin and 130.0 ng of hesperetin (Fig. 3B). This alkaline solvent system separated naringenin and its internal standard, hesperetin, from interfering materials present in the urine. For the determination of naringenin, therefore, the solvent system containing a very small amount of triethylamine and hesperetin as the internal standard were used. It was also found that the wavelength of maximum UV absorbance at 292 nm for naringenin in the acidic mobile phase was shifted to 324 nm. The peak intensity at the absorbance of 324 nm was about 1.5 times higher than that at 292 nm. A small peak (peak a) observed at the retention time of naringenin in Fig. 3A was also detected. Presently, whether or not this peak results from the endogenous naringenin has not been confirmed. This compound was detected in all 24 h urine samples freshly collected from five healthy volunteers. Peak-height ratios of this compound to 130.0 ng of hesperetin in 1.0 ml aliquots of urine samples on the chromatogram were 0.088±0.061 (mean±S.D.), corresponding to about 15 ng of naringenin.

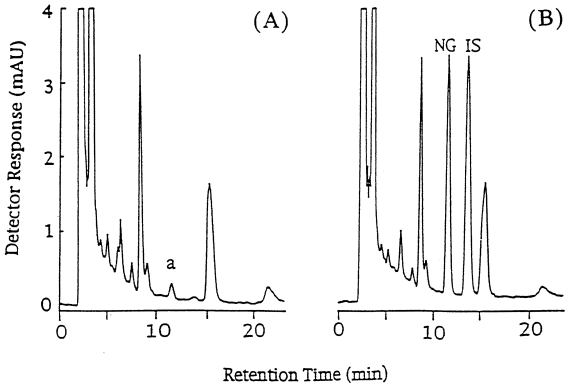

Fig. 3. HPLC chromatograms analyzed blank urine (A), and (B) urine spiked with naringenin (112.5 ng) and hesperetin (130.0 ng). Chromatographic conditions were described in the text. Peaks: NG=naringenin; IS=hesperetin.

Calibration graphs were prepared by using blank urine to which naringin and naringenin had been added in the ranges of 35–210 ng and 20–160 ng, respectively, and hesperidin (166.3 ng) or hesperetin (130.0 ng) as the internal standard. The peak-height 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-height ratios (y) and the mixed mass ratios (x). A least-squares regression analysis gave typical regression lines $y=0.0064x-0.0325$ ($r=0.999$) for naringin and $y=0.0081x+0.1184$ ($r=0.999$) for naringenin.

The accuracy of measurements was determined by adding known amounts of naringin or naringenin to 1.0 ml aliquots of 24 h urine. To the urine samples were added fixed amounts of hesperidin (166.3 ng) or hesperetin (130.0 ng) and different amounts of naringin (23.8, 47.5 and 142.5 ng) or naringenin (22.5, 45.0 and 135.0 ng). Table 1 shows that the amounts of naringin and naringenin added were in good agreement with the amounts of naringin and naringenin measured, the relative errors being less than 5% for naringin (47.5 and 142.5 ng/ml), 20% for 23.8 ng/ml of naringin and ±8% for naringenin. The inter-assay relative standard deviations (R.S.Ds.) ($n=6$) were less than 9% for naringin and less than 8% for naringenin. The intra-assay of R.S.Ds. ($n=6$) were less than 9% for naringin (23.8, 47.5 and 142.5 ng/ml) and 10% for naringenin (22.5, 45.0 and 135.0 ng/ml). The precision (R.S.D.) and the accuracy (R.E.) at the limit of quantification were acceptable in view of the international recommendations [18]. The lower limits of quantification were judged to be ca. 25 ng/ml for naringin and naringenin. The sensitivity of the present HPLC assay (defined as a signal-to-noise ratio of about 3) was 4.75 ng/ml urine for naringin. For naringenin, since urine samples contained an interfering peak a on the chromatogram (Fig. 3A), the sensitivity was examined using water samples and was determined to be 1.08 ng/ml.

Naringin and naringenin added to urine samples were found to be stable for at least 24 h at 37°C. When stored at −20°C, both compounds added to urine were also stable under the storage conditions for at least 3 months.

As an example of the application of this method, the urinary excretion of naringin, naringenin and naringenin glucuronides were investigated in the

Table 1
Accuracy of HPLC determination of naringin and naringenin in human urine

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values						Mean±S.D.		
Naringin									
23.8 ng	31.6	30.3	27.0	25.4	29.2	25.8	28.2±2.54	+18.4	9.0
47.5 ng	50.5	49.5	49.2	47.8	51.0	50.4	49.7±1.11	+4.6	2.2
142.5 ng	149.3	144.7	147.2	145.9	143.2	150.1	146.7±2.66	+2.9	1.8
Naringenin									
22.5 ng	20.3	19.1	24.2	20.4	21.2	19.8	20.8±1.79	−7.6	8.0
45.0 ng	40.5	43.9	41.8	43.9	45.8	42.8	43.1±1.85	−4.3	4.6
135.0 ng	139.2	130.0	131.5	142.0	137.3	135.5	135.9±4.57	+0.7	3.4

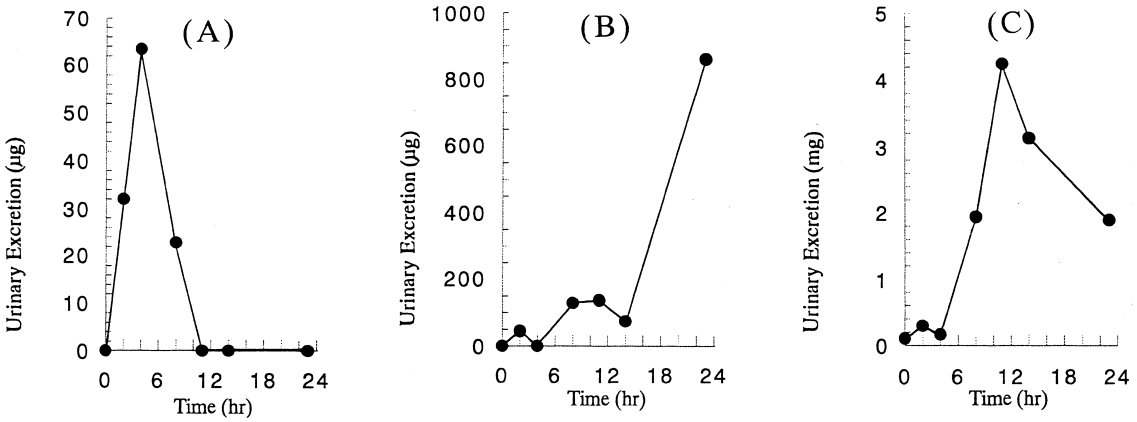

Fig. 4. Urinary excretion of naringin (A), naringenin (B) and naringenin glucuronides (C).

urine from a healthy volunteer who received orally 500 mg of naringin. Fig. 4 shows the cumulative urinary excretion of naringin (A), naringenin (B) and naringenin glucuronides (C). The present method can be applied to the pharmacokinetic study after administering naringin to human. Furthermore, as shown in Fig. 4A, it is interesting to note that naringin itself was also found in the urine sample. Further detailed investigation on the identification of naringin in human urine will be reported elsewhere.

Acknowledgements

This work was supported, in part, by a Project Research Grant of Kyorin University.

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