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Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations

Feras Imad Kanaze, Chrysi Gabrieli, Eugene Kokkalou, Manolis Georgarakis, Ioannis Niopas *

Department of Pharmacy, School of Health Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

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Abstract

Diosmin, hesperidin and naringin are flavonoid glycosides that occur naturally in citrus fruits. They exert a variety of pharmacological properties such as anti-inflammatory, antioxidant and free radical scavenging and antiulcer effects and also inhibit selected cytochrome P-450 enzymes resulting in drug interactions. A reversed-phase high-performance liquid chromatographic method has been developed for the simultaneous determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical preparations. Diosmin, hesperidin, naringin and the internal standard rhoifolin were separated using tetrahydrofuran/water/acetic acid (21:77:2, v/v/v) as the mobile phase at 34 °C, using a C8 reversed-phase column. The method was linear in the 0.25–20.0 μg/ml concentration range for all three flavonoid glycosides ($r > 0.999$). The method has been successfully applied to the determination of all three flavonoid glycosides in several samples of different citrus fruit juices sold in Greece and for the determination of diosmin and hesperidin in pharmaceutical preparations.

Keywords: Diosmin; Hesperidin; Naringin; Citrus flavonoids; HPLC determination

1. Introduction

Flavonoids are important polyphenolic secondary metabolites that are widely distributed in medicinal plants and foods of plant origin providing much of the flavor and color to fruits and vegetables [1]. They usually occur in plants as glycosides and they are categorized into flavones, flavonols and flavanones [2]. Flavonoids have been found to exert various pharmacological activities, such as anti-inflammatory [3], antioxidant and free radical scavenging [4], antiulcer [5] and antiallergenic [6]. Several epidemiological studies indicate an inverse association between

* Corresponding author. Tel.: +30-2310-99-7663; fax: +30-2310-99-7645.
E-mail address: niopas@pharm.auth.gr (I. Niopas).

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the intake of flavonoids and the risk of cardiovascular diseases [7–9] and different types of cancer [10].

The flavone glycoside diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside) is common constituent in many citrus species [11], its flavanone analog hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglycoside) is the primary flavonoid glycoside in orange (*Citrus sinensis*) and lemon (*Citrus limonium*) [12] and the flavanone glycoside naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglycoside) is the predominant flavonoid in grapefruit (*Citrus paradisi*), contributing to its bitter flavor [13]. Flavonoid glycosides have been used to detect admixtures of citrus juices such as adulteration of orange juice by grapefruit juice [14]. Diosmin, hesperidin and naringin possess antioxidant [15], blood lipid lowering [16] and anti-carcinogenic activities [17]. Diosmin and hesperidin improve venous tone, enhance microcirculation, assist healing of venous ulcers and they are used for the treatment of chronic venous insufficiency [18], haemorrhoids [19] and the prevention of postoperative thromboembolism [20]. Naringin inhibits selected cytochrome P-450 enzymes, such as CYP1A2 and CYP3A4, and thus alters the pharmacokinetics of a variety of clinically used drugs resulting in drug interactions [21].

Reversed-phase high-performance liquid chromatography combined with different detectors is the commonly used analytical method for separation of flavonoids [22–25]. Recently a number of HPLC methods have been published for the separation and determination of diosmin, hesperidin and naringin, either alone or in combination with other flavonoid glycosides in plant extracts, biological fluids, or pharmaceutical formulations [26–33]. However, some of these methods are not validated, some are time-consuming, some require expensive instruments or laborious extraction techniques, or there were no internal standard. No method was found in the literature for the simultaneous determination of all three flavonoid glycosides mentioned.

The purpose of this investigation was to develop a novel HPLC method for the simultaneous quantitative determination of diosmin, hesperidin and naringin, suitable for the analysis of these flavonoid glycosides in citrus fruit juices and in pharmaceutical preparations.

2. Experimental

2.1. Chemicals and reagents

Diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside), naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglycoside) and internal standard rhoifolin (apigenin 7-*O*-neohesperidoside) were supplied from Sigma (St. Louis, MO, USA). Hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglycoside) was purchased from Acros Organics (New Jersey, USA). HPLC-grade acetonitrile, methanol, tetrahydrofuran, dimethyl sulfoxide (DMSO) and acetic acid were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

2.2. Chromatographic conditions

The analyses were performed using a high-performance liquid chromatographic system (Varian, Palo Alto, CA, USA) consisting of a solvent delivery pump (Model 2510), a variable wavelength UV-Vis detector (Model 2550), a manual injector with a 20-μl loop (Rheodyne, Cotati, CA, USA) and an integrator (Model 4290). Separation was performed on a Macherey Nagel Nucleosil C8 analytical column (250×4.6 mm I.D., 5 μm particle size) (Rigas Labs, Thessaloniki, Greece), proceeded by a guard column (20×4.6 mm I.D.) dry packed with pellicular ODS material (37–53 μm) (Whatman, Kent, UK).

The mobile phase consisted of tetrahydrofuran/water/acetic acid (21:77:2, v/v/v) and was filtered through a 0.45-μm pore size nylon filter (Alltech, Deerfield, IL, USA) and degassed by ultrasonic treatment before use. The HPLC system was operated isocratically at a flow rate of 0.85 ml/min at 34 °C and the detector was set at 280 nm. The integrator attenuation was 8 and the chart speed was 0.1 cm/min.

2.3. Standard solutions

Stock solutions of diosmin, hesperidin, naringin and the internal standard rhoifolin were prepared separately by dissolving appropriate amounts of the compounds in methanol/DMSO (1:1) to achieve concentrations of 400 μg/ml for each compound. Calibration standard samples containing diosmin, hesperidin and naringin each at 0.25, 0.5, 1.0, 2.0, 4.0, 10.0, and 20.0 μg/ml and rhoifolin as internal standard at 20.0 μg/ml were freshly prepared by appropriate dilutions with methanol/DMSO (1:1) from the stock solutions.

2.4. Sample preparation

Commercial brands of citrus fruit juices as well as fresh citrus fruits such as oranges, lemons, tangerines, and grapefruits from different varieties, were purchased from supermarkets in Thessaloniki (Greece) during the winter of 2003. Commercial citrus fruit juices and freshly prepared hand-squeezed citrus fruit juices were sonicated at room temperature for 15 min and filtered before use. To 50 μl of citrus fruit juice samples, 50 μl of internal standard solution containing 400 μg/ml and 900 μl of methanol were added. The contents were vortex-mixed for 30 s, centrifuged at 2000 × *g* for 10 min and a 20-μl aliquot was injected into the HPLC system for quantification.

Daflon® tablet (Les Laboratoires Servier, Gidy, France) is a mixture of micronized flavonoid glycosides consisting of 450 mg of diosmin and flavonoid extract equivalent to 50 mg of hesperidin. Five Daflon® tablets were accurately weighted and finely powdered. A weight equivalent to one tablet was sonicated at 30 °C with 90 ml of methanol/DMSO (1:1) for 30 min. The extract was filtered and the volume was adjusted to 100 ml with methanol. To 50 μl of this solution 1 ml of internal standard solution containing 400 μg/ml was added, and then the volume made up to 20 ml with water and after vortex-mixed for 30 s, a 20-μl volume was injected into the HPLC system for analysis.

2.5. Assay validation

The linearity of the assay was demonstrated over the concentration range 0.25–20 μg/ml of diosmin, hesperidin, and naringin by assaying calibration standards in triplicate at seven separate concentrations on three separate occasions. Calibration curves were obtained by plotting the peak height ratios of diosmin/internal standard, hesperidin/internal standard, or naringin/internal standard versus concentrations of added diosmin, hesperidin, or naringin.

Within-day assay precision and accuracy were determined in conjunction with the linearity studies by assaying five control samples at each of three concentrations (2, 8, and 15 μg/ml). Concentrations of each of the three flavonoid glycosides in control samples were determined by application of the obtained standard curve. Assay precision was assessed by calculating the %R.S.D. at each concentration and assay accuracy was determined by calculating the estimated concentrations as a percent of the nominal concentrations.

3. Results and discussion

3.1. Optimization of separation conditions

The identification of flavonoid glycosides diosmin, hesperidin, and naringin was made by comparing their retention times and UV spectra with those of standards. Several reversed-phase HPLC methods using various mixtures of acetonitrile/water or methanol/water with acetic or phosphoric acid have been used for the analysis of flavonoid glycosides in crude plant materials, food products and pharmaceutical formulations [22–33]. To our knowledge, no literature method is available for the simultaneous determination of diosmin, hesperidin, and naringin. Therefore, using a univariate method of optimization, a different mobile phase consisting of tetrahydrofuran and water was tested in order to achieve optimal separation.

The chromatographic conditions described in this method were achieved at after investigating

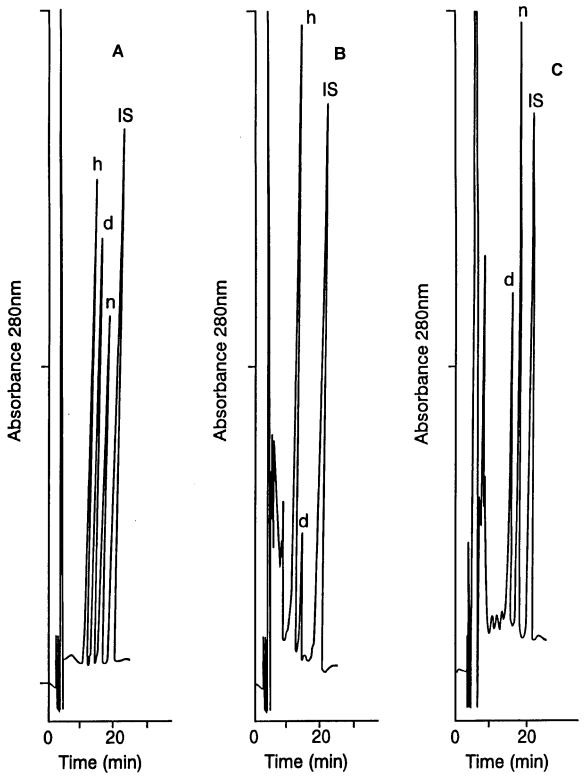

Fig. 1. Examples of HPLC chromatograms: (A) calibration standards; (B) commercial orange fruit juice sample; (C) hand-squeezed grapefruit juice sample. Peaks: (d) diosmin; (h) hesperidin; (n) naringin; (IS) rhoifolin (internal standard).

C18 or C8 reversed-phase columns and several mobile phases consisting of mixtures of acetonitrile, methanol or tetrahydrofuran and water in different ratios. Regarding the selection of the reversed-phase column and the mobile phase, optimum chromatographic separation of diosmin, hesperidin, naringin, and internal standard were achieved using a C8 reversed-phase column with tetrahydrofuran instead of acetonitrile or methanol. The addition of acetic acid in the solvent system (tetrahydrofuran/water/acetic acid 21:77:2, v/v/v/) which suppresses the ionization of phenol groups lead to better separation of all three flavonoid glycosides and internal standard. The separation was further improved when column temperature was increased to 34 °C.

Symmetrical, sharp and well-resolved peaks were observed for diosmin, hesperidin, naringin, and internal standard. Typical chromatograms obtained from calibration standards, orange fruit juice and grapefruit juice samples are shown in Fig. 1. There were no peaks due to other minor coextracted materials interfering with diosmin, hesperidin, naringin, or internal standard. The retention times for hesperidin, diosmin, naringin, and internal standard were 12.0, 14.0, 16.6, and 20.1 min, respectively.

Table 1
Within-day accuracy and precision for diosmin, hesperidin and naringin in control samples

Nominal concentration (μg/ml)	Mean found concentration ($n = 5$, μg/ml)	Accuracy[a]	Precision[b] (%R.S.D.)
Diosmin			
2	2.17	108.5	3.8
8	7.6	95.0	1.1
15	15.4	102.8	0.8
Hesperidin			
2	1.97	98.5	1.6
8	7.9	98.8	1.2
15	15.3	102.0	0.9
Naringin			
2	2.16	108.0	3.6
8	7.5	93.8	1.8
15	15.1	100.7	0.7

[a] Accuracy: found concentration expressed in % of the nominal concentration.
[b] R.S.D., relative standard deviation.

Table 2
Concentrations of diosmin, hesperidin, and naringin in commercial and hand-squeezed citrus fruit juice samples

Citrus fruit juice samples	Diosmin (μg/ml)	Hesperidin (μg/ml)	Naringin (μg/ml)
Orange			
1	22.9	141.8	–
2	64.4	443.0	–
3	45.9	247.0	–
4	26.4	207.1	–
5	7.9	53.8	–
6	26.4	217.2	–
7	21.9	102.9	–
8	22.7	138.5	–
9	72.3	735.3	2.3
10	35.7	179.5	–
Mean ± S.D.	34.7 ± 20.3	246.6 ± 201.3	
Range	7.9–72.3	53.8–735.3	
Tangerine			
1	9.6	52.1	–
2	6.7	45.9	–
3	21.2	48.9	–
4	7.4	86.1	1.2
5	14.4	170.5	0.5
6	15.7	237.7	0.7
Mean ± S.D.	12.5 ± 5.6	166.7 ± 79.7	0.8 ± 0.4
Range	6.7–21.2	45.9–237.7	0.5–1.2
Lemon			
1	1.6	15.9	–
2	11.9	47.2	3.8
3	17.8	28.8	–
Mean ± S.D.	10.4 ± 8.2	30.6 ± 15.7	
Range	1.6–17.8	15.9–47.2	
Grapefruit			
1	1.9	164.0	584.7
2	13.7	43.9	351.3

3.2. Validation

The linearity of the method was confirmed for concentrations ranging from 0.25 to 20 μg/ml for all three flavonoid glycosides. Their equations were calculated by using linear regression analysis. Calibration curves were established on each day of analysis and typical calibration curves had the regression equations of $y = -0.00234 + 0.07627x$, $y = -0.01459 + 0.08256x$, and $y = -0.00412 + 0.06206x$ ($r > 0.99$) for diosmin, hesperidin, and naringin, respectively.

Within-day precision for diosmin, hesperidin, and naringin was equal to or less than 3.8, 1.6, and 3.6% for control samples containing 2, 8, and 15 μg/ml, respectively. Within-day accuracy was better than 108.5, 102.0, and 108.0% for diosmin, hesperidin, and naringin, respectively (Table 1).

The limit of quantitation, defined as the lowest quantifiable concentration on the calibration curve at which both precision and accuracy were within the maximum tolerable CV of 15%, was deemed to be 0.1 μg/ml for each flavonoid glycoside.

3.3. Analysis of diosmin, hesperidin, and naringin in citrus fruit juice samples

The concentrations of diosmin, hesperidin, and naringin found in commercial and hand-squeezed citrus fruit juice samples are shown in Table 2. The

Table 3
Determination of diosmin and hesperidin in Daflon® tablets

Flavonoid glycoside[a]	Label claim (mg)	Found ± S.D.	Percentage of label claim ± %R.S.D.
Diosmin	450	448.2 ± 3.2	99.6 ± 0.7
Hesperidin	50	41.7 ± 2.6	83.3 ± 1.6

[a] Results are the average of five separate determinations.

results proved to be variable. Diosmin was detected in all citrus fruit juice samples analyzed. Hesperidin was the major flavonoid glycoside found in orange, tangerine and lemon fruit juice samples. The mean ± S.D. concentration of hesperidin was 246.6 ± 201.3 μg/ml (range 53.8–735.3 μg/ml), 166.7 ± 79.7 μg/ml (range 45.9–237.7 μg/ml) and 30.6 ± 15.7 μg/ml (range 15.9–47.2 μg/ml) for orange, tangerine and lemon juice samples, respectively. Naringin was the predominant flavonoid detected in grapefruit juice samples, while hesperidin was found to be present in very small concentrations. In agreement with previous literature data [14], naringin was not detected in any of the orange fruit juice samples analyzed except in one sample, indicating possible adulteration of this sample by grapefruit juice. The concentrations of naringin and hesperidin in citrus juice samples analyzed were similar to those reported previously by other authors [28,32,34].

3.4. Determination of diosmin and hesperidin in Daflon® tablets

Diosmin and hesperidin are characterized by poor water solubility, which is responsible for their low bioavailability following oral administration and presents difficulties for extraction from crude plant materials and pharmaceutical preparations [35]. Sonication of the powdered Daflon® tablets with methanol/DMSO (1:1) at 30 °C for 30 min was found to be a very efficient system for the complete extraction of diosmin and hesperidin from Daflon® tablets. Table 3 shows the results of five separate determinations of diosmin and hesperidin in Daflon® tablets. The mean ± %R.S.D. potency of diosmin and hesperidin were found to be 99.6 ± 0.7 and 83.3 ± 1.6%, respectively.

3.5. Conclusion

A reversed-phase HPLC method for the simultaneous determination of diosmin, hesperidin and naringin was developed. The method was simple, specific, precise and accurate and was successfully used for the quantitative analysis of diosmin, hesperidin and naringin in citrus fruit juice samples and of diosmin and hesperidin in pharmaceutical formulations.

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