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Short communication

High-performance liquid chromatographic analysis of polyhydroxyflavones using solid-phase borate-complex extraction

Hironori Tsuchiya*

Department of Dental Pharmacology, Asahi University School of Dentistry, 1851 Hozumi, Hozumi-cho, Motosu-gun, Gifu 501-0296, Japan

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Abstract

A high-performance liquid chromatographic method using a solid-phase borate-complex extraction pretreatment was studied for the selective quantitation of polyhydroxyflavones in vegetables, red wine and human blood plasma. Homogenate, extract and intact samples were applied to phenylboric acid cartridges to retain polyhydroxyflavones on the solid-phase by forming the borate-complex, followed by elution of the retained analytes with an acidic solvent. Reversed-phase chromatography with diode array detection allowed the simultaneous separation of rutin, myricetin, fisetin, morin, quercetin and kaempferol without significant interference from other components, indicating high selectivity of the solid-phase borate-complex extraction. The absolute recoveries of quercetin, fisetin and rutin were superior to those of kaempferol, myricetin and morin, suggesting a difference in the complex formation efficiency between 1,2- and 1,3-diol structures. When using fisetin as an internal standard, polyhydroxyflavones were quantified in the concentration range 0.10–30.0 μg/ml. In replicate spiking experiments with standards, the mean relative recoveries ranged between 75.7 and 104.6%, and the intra- and inter-assay C.V.s ranged between 0.8 and 10.2% for onion, wine and plasma samples. The proposed method will be applicable to nutritional and pharmacokinetic experiments of polyhydroxyflavones.

Keywords: Polyhydroxyflavones; Borate; Rutin; Myricetin; Fisetin; Morin; Quercetin; Kaempferol

1. Introduction

Polyhydroxyflavones are contained in various plants and plant products including vegetables and fruits, and their considerable amounts are estimated to be daily ingested via dietary sources [1–3]. Quercetin, kaempferol, fisetin, rutin, myricetin and morin are known as the most common dietary polyhydroxyflavones (Fig. 1). For these bioflavonoids, there is accumulating evidence to suggest a wide range of pharmacological activities, such as anti-inflammatory, antiallergic, antimicrobial, antioxidative, enzyme-inhibitory effects, etc. [4,5]. Among anticancer phytochemicals in food [6], quercetin has been reported to exert the intensive antiproliferative activity against various tumor cells, leading to its potent clinical use [7–9]. The pharmacological significance of polyhydroxyflavones interests researchers concerning their content in diets and bioavailability in humans.

In nutritional and pharmacokinetic studies, a

*Tel.: +81-58329-1432; fax: +81-58329-1432; e-mail: hiro@dent.asahi-u.ac.jp

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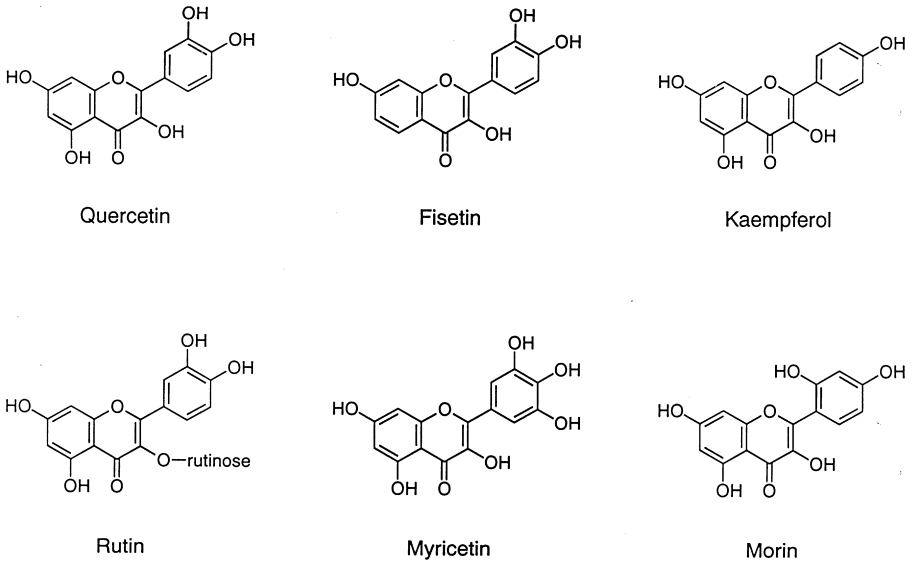

Fig. 1. Structures of polyhydroxyflavones.

separative analytical technique is most suitable for the simultaneous quantitation of different polyhydroxyflavones. Several methods by high-performance liquid chromatography (HPLC) have been recently applied to the analysis of vegetables, fruits and beverages [10–12], and blood plasma [13]. However, they are not necessarily satisfactory in analytical quality, especially specificity to analytes. While the purification of biological samples prior to HPLC separation is the determinant for success in quantitative analysis, a selective pretreatment method has not been available for polyhydroxyflavones. Flavones consist of a benzene ring (A) condensed with a γ-pyrone ring (C) possessing a phenyl ring (B) at the 2-position. The pharmacologically interesting polyhydroxyflavones have 1,2- and/or 1,3-dihydroxyl groups in the B and/or A ring (Fig. 1). Such a diol structure potentially forms the complex with appropriate ligands which is usable for selective purification. Based on that concept, several catechin derivatives belonging to the related bioflavonoids were recently pretreated using a complex formation with either alumina or diphenylborate, resulting in selective HPLC analysis [14,15].

In the present study, six kinds of polyhydroxyflavones were purified by a solid-phase borate-complex extraction, and then analyzed by reversed-phase HPLC with diode array detection. After optimization of different conditions, the method was applied to the analysis of vegetables, red wine and human plasma.

2. Experimental

2.1. Chemicals and materials

Quercetin, kaempferol, fisetin, rutin and morin were purchased from Tokyo Kasei (Tokyo, Japan), except for myricetin which was from Aldrich (Milwaukee, WI, USA). Their stock solutions (1.0 mg/ml of each) were prepared every month by dissolving the standards in ethanol or 50% ethanol aqueous solution, and they were stored at 4°C. They were diluted as required with water before use. Varian Bond Elut PBA (phenylboric acid, 100 mg/ml) cartridges (Harbor City, CA, USA) were used for a solid-phase extraction. Acetonitrile of HPLC grade from Kishida (Osaka, Japan) was used. All other reagents were of the highest analytical grade available. Water was redistilled by an all-glass apparatus after purification by a Milli-RO water purification system (Nihon Millipore, Tokyo, Japan).

Vegetables and red wine were purchased from commercial outlets. The colored outer skins of onions were powdered using a mortar and pestle.

After the powders were homogenized in 50% (v/v) ethanol aqueous solution and centrifuged at approximately 10 000 *g* for 10 min, the obtained extracts (50 mg/ml) were used for analysis. The edible parts of onions and other vegetables were minced, homogenized and centrifuged at approximately 10 000 *g* for 20 min, and the obtained supernatants were used for analysis. When any precipitates were found, wine was filtered through a pore size of 0.22 μm and the filtrates were used for analysis after diluting with water as required. In blood analysis, quercetin (0.50–5.00 μg/ml) corresponding to the concentration found in an administration experiment [13] was added to human heparinised plasma.

All of the glassware used for quantitative analyses was silanized to avoid the adsorption of analytes onto the glass surfaces [16].

2.2. Sample preparation

Before loading a sample solution, a PBA cartridge was washed successively with 1 ml of 28% (v/v) acetonitrile aqueous solution containing 1% (v/v) trifluoroacetic acid, 1 ml of water and 1 ml of 0.5 *M* potassium phosphate buffer (pH 8.5). The mixture of 50 μl of onion, wine or plasma sample, 50 μl of an aqueous fisetin solution (4.00 μg/ml) as an internal standard, 150 μl of water and 250 μl of 1.0 *M* potassium phosphate buffer (pH 8.5) was loaded to the cartridge. Following a wash with 1 ml of 10 m*M* potassium phosphate buffer (pH 8.5), analytes were eluted with 2 ml (0.5 ml×4) of 28% (v/v) acetonitrile aqueous solution containing 1% (v/v) trifluoroacetic acid. A 100-μl aliquot of eluates was subjected to HPLC separation.

2.3. HPLC analysis

The HPLC system consisted of an LC-10ADVP liquid chromatograph (Shimadzu, Kyoto, Japan), a KMT-60-AII autosampler (Kyowa Seimitsu, Tokyo, Japan), a Shim-pack CLC-C8 (M) column (250×4.6 mm I.D., particle size 5 μm, Shimadzu) placed in a thermo-controller (Kyowa Seimitsu) and an SPD-M10AVP diode array detector (Shimadzu) controlled by an FMV-5133D5 personal computer (Fujitsu, Tokyo, Japan). The separation was performed by an isocratic elution using the mobile phase, a mixture of trifluoroacetic acid, acetonitrile and water (1:28:71, v/v/v), which was delivered at a flow-rate of 1.0 ml/min and at a column temperature of 50°C. In routine analyses, eluates from the column were detected at 370 nm. Polyhydroxyflavones were quantified based on the calibration graphs prepared as described in Section 2.4 and their concentrations were corrected by the recoveries obtained from spiking experiments.

2.4. Analytical evaluation

To evaluate the quantitative range, calibration graphs were prepared by plotting peak area ratios of polyhydroxyflavones to fisetin against the known concentrations. Rutin, myricetin, morin, quercetin and kaempferol (0.050–30.0 μg/ml of each) were treated as described in Sections 2.2 and 2.3. The mean ratios of triplicate experiments were plotted.

To evaluate the recovery and analytical precision, standard myricetin, morin, quercetin and/or kaempferol (0.50 or 5.00 μg/ml of each) were added to wine, onion homogenate, onion skin extract and plasma samples. Replicate spiked solutions were analyzed as described in Sections 2.2 and 2.3. The mean recovery ($n=6$), and intra- (analyzed on the same day, $n=6$) and inter-assay C.V. (analyzed on different days, $n=4$) were determined.

3. Results and discussion

Catechins with diol structures similar to those in polyhydroxyflavones were recently purified by liquid-phase extraction using a complex formation with diphenylborate [15,17]. In that method, catechins are back-extracted to the acidic aqueous phase after a liquid–liquid extraction of the catechin–diphenylborate complex with an organic solvent. It was impossible, however, for the tested polyhydroxyflavones to perform the borate-complex extraction in liquid-phase because of their low back-extractability to the aqueous phase. Therefore, the solid-phase extraction using a PBA cartridge was employed in the present study. Polyhydroxyflavones were retained on the cartridge by forming the complex at pH 8.5, thereafter they were eluted with a mixture of trifluoroacetic acid, acetonitrile and water, followed by

HPLC analysis. The elution profile based on peak areas showed that all of the retained rutin and myricetin were eluted with 1 ml (0.5 ml×2) of an acidic solvent, while almost complete elution for the others (> 90% of the retained ones) needed the solvent volume of at least 2 ml (0.5 ml×4).

Standard polyhydroxyflavones (5.00 μg/ml of each) were chromatographed after pretreatment by the solid-phase borate-complex extraction. The result is shown in Fig. 2A. The absolute recoveries, which were estimated by comparing the obtained peak areas with the directly (without pretreatment) injected standards of corresponding concentrations, were as follows: 105.6% for rutin, 39.9% for myricetin, 93.0% for fisetin, 69.8% for morin, 93.3% for quercetin and 80.5% for kaempferol. Quercetin, fisetin and rutin showing a recovery over 90% have 1,2-dihydroxyl groups in the B ring, whereas kaempferol and morin with relatively low recovery have 1,3-dihydroxyl groups in the A and/or B ring (Fig. 1). Different recoveries suggest a difference in complexing efficiency between 1,2- and 1,3-diol structures. Myricetin exceptionally showed much lower recovery, for which the occurrence of steric hindrance by an additional hydroxyl group and the unstability of triol flavonoids may be responsible [17,18].

Onion and red wine are well-known to contain considerable amounts of several polyhydroxyflavones [1,10–12]. Quercetin has been studied as an anticancer agent by a cell growth inhibition experiment [7] and a phase I clinical trial [13]. Onion, red wine and spiked plasma samples were subjected to the solid-phase borate-complex extraction, followed by HPLC separation. The results are shown in Fig. 2B–D. The detected quercetin, myricetin, morin and/or kaempferol were identified by comparing diode array spectra and capacity factors of the obtained peaks with standards. Major interfering peaks did not appear on chromatograms of vegetable samples. Quercetin was not detected in any plasma samples obtained from the subjects who had consumed neither red wine nor onion for at least 8 h prior to the blood collection. When standard quercetin (5.00 μg/ml) was added to the blank plasma, the corresponding peak appeared on chromatograms. In contrast to a previous HPLC method [13], plasma samples provided no peaks other than the added quercetin, which is attributed to high selectivity of the solid-phase borate-complex extraction. In analysis of red wine, several large peaks observed at shorter retention time may be derived from catechins and their related polyhydroxy flavonoids that are contained at high concentrations in wine [1,12] because they possibly form the borate-complex.

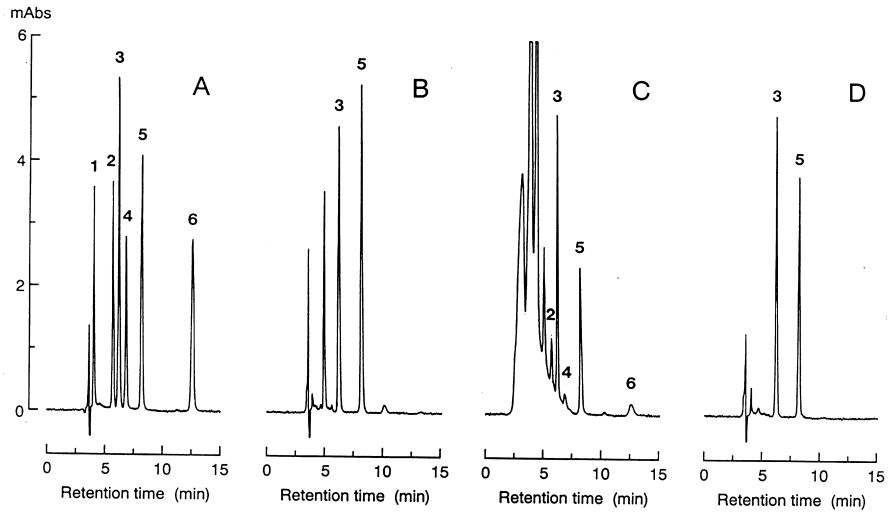

Fig. 2. Chromatograms obtained from standard, onion, wine and plasma samples. (A) standard polyhydroxyflavones (5.00 μg/ml of each), (B) onion skin extract, (C) wine and (D) plasma spiked with quercetin (5.00 μg/ml). Detection at 370 nm. Peaks: (1) rutin; (2) myricetin; (3) fisetin (internal standard); (4) morin; (5) quercetin; and (6) kaempferol.

Since no peaks were detected at the retention time of fisetin in analyses of all the tested samples, it was used as an internal standard. The calibration graphs showed the quantitative range 0.10–30.0 μg/ml for quercetin and rutin, and 0.20–30.0 μg/ml for the others. The regression equations were as follows: $y=0.136x+0.142$ ($r^2=0.998$) for rutin, $y=0.062x-0.035$ ($r^2=0.988$) for myricetin, $y=0.093x+0.028$ ($r^2=0.995$) for morin, $y=0.276x-0.064$ ($r^2=0.999$) for quercetin, and $y=0.122x-0.053$ ($r^2=0.997$) for kaempferol. Such quantitative sensitivity is comparable to or 2-fold higher than previous methods [10,13].

Recovery and analytical reproducibility were evaluated by spiking the standards (0.50 and 5.00 μg/ml) almost corresponding to the natural concentrations in red wine and onion samples. In a phase I clinical study, quercetin was found to show the concentration range 6–12 μg/ml in human plasma 15 min after administration of an intravenous dose of quercetin (945 mg/m^2) in DMSO vehicle [13]. Therefore, standard quercetin of 0.50 and 5.00 μg/ml was spiked to plasma samples to evaluate the recovery and analytical precision. The mean relative recoveries, and intra- and inter-assay C.V.s are shown in Table 1. The recovery from plasma samples tended to be lower than those from wine and onion, which is ascribable to the influence of plasma proteins. The obtained values are almost comparable to those of previous methods [10–13].

Application of the proposed method to onions ($n=3$) revealed that the contents of quercetin were 127.9–144.9 μg/g in outer skin and 3.89–4.04 μg/g in edible part. The concentrations in different brands of red wine ($n=10$) ranged from <0.10 to 6.83 μg/ml for quercetin, from 2.09 to 15.45 μg/ml for myricetin, from <0.20 to 1.82 μg/ml for morin and from <0.20 to 1.18 μg/ml for kaempferol. Although other vegetables and fruits have been also reported to contain polyhydroxyflavones [1], the tested broccoli, lettuce, garlic and apple provided no significant peaks corresponding to quercetin, kaempferol, myricetin, morin and rutin. These quantitative results partly agree with or are different from those in previous reports [11,12]. Differences found may be due to varietal, seasonal and/or brand differences in the analyzed samples.

The proposed method will be a useful tool for the quantitative analysis of polyhydroxyflavones in nutritional and pharmacokinetic studies.

Table 1
Recovery and analytical precisions[a]

Sample	Spiked standard	Mean recovery (%)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
Red wine	Myricetin (5.00 μg/ml)	91.2	4.1	4.9
	Morin (5.00 μg/ml)	95.8	5.0	5.3
	Quercetin (5.00 μg/ml)	94.8	0.8	1.0
	Kaempferol (5.00 μg/ml)	97.2	4.4	5.2
Red wine	Myricetin (0.50 μg/ml)	81.5	7.2	10.2
	Morin (0.50 μg/ml)	86.8	7.1	9.8
	Quercetin (0.50 μg/ml)	92.0	5.2	6.2
	Kaempferol (0.50 μg/ml)	96.0	8.2	8.5
Onion edible part homogenate	Quercetin (5.00 μg/ml)	103.8	1.8	2.2
Onion outer skin extract	Quercetin (5.00 μg/ml)	104.6	2.9	3.5
Human plasma	Quercetin (5.00 μg/ml)	82.8	4.9	5.6
Human plasma	Quercetin (0.50 μg/ml)	75.7	6.5	7.8

[a] Mean recovery and reproducibility were evaluated by analyzing replicate samples spiked with standard polyhydroxyflavones: $n=6$ for recovery, $n=6$ for intra-assay precision and $n=4$ for inter-assay precision.

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