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Short Communication

Determination of rhoifolin and daidzin in human plasma by high-performance liquid chromatography

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Abstract

A method for determining flavonoids in human plasma is presented for application to pharmacokinetic studies of two flavonoids, rhoifolin and daidzin. Isocratic reversed-phase high-performance liquid chromatography (HPLC) was used with genistin as an internal standard and solid-phase extraction using a Sep-Pak C_{18} cartridge. The mobile phases were acetonitrile–0.1 *M* ammonium acetate solution (20:80, v/v) for rhoifolin and methanol–0.1 *M* ammonium acetate solution (33:67, v/v) for daidzin. The detection limits on-column were 2 ng for rhoifolin and 0.5 ng for daidzin.

1. Introduction

Flavonoids are naturally occurring compounds ubiquitous in the plant kingdom. These compounds have been known to show a variety of biological and pharmacological activities such as the inhibition of enzymes [1,2], free radical scavenging [3] and anti-inflammation [4]. Recently, it has been reported that several flavonoids such as rhoifolin and daidzin (Fig. 1), which possess the glucoside moiety at the 7-position of the flavonoid skeleton, activate polymorphonuclear leukocytes (PMN) and induce the cytotoxic activity of PMN against tumour cells *in vitro*. The position and identity of the sugar moiety have been shown to be of significant importance in activating PMN [5].

In order to conduct clinical pharmacokinetic studies on rhoifolin and daidzin, methods for their determination in human plasma by HPLC were developed and are described here.

2. Experimental

2.1. Chemicals and reagents

Rhoifolin (4′,5,7-trihydroxyflavone-7-rhamnoglucoside), daidzin (4′,7-dihydroxyisoflavone-7-glucoside) and genistin (4′,5,7-trihydroxyisofla-

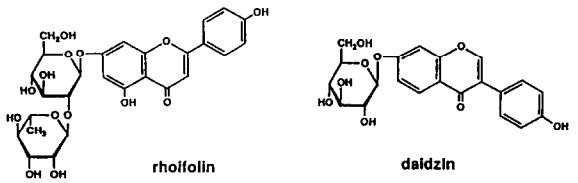

Fig. 1. Structures of rhoifolin and daidzin.

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vone 7-glucoside) were purchased from Extrasynthase (Genay, France). Stock standard solutions of the flavonoids in methanol were prepared. All analyses were performed by appropriately diluting the stock standard solutions with water. All other chemicals and solvents were of analytical-reagent grade and used as received.

2.2. Sample preparation

To 1.0-ml aliquots of human plasma were added different amounts of rhoifolin or daidzin in 30–120 μl of standard solutions and a fixed amount of genistin as an internal standard (for rhoifolin, 103.5 ng dissolved in 50 μl of water; for daidzin, 144.9 ng dissolved in 70 μl of water). The plasma sample was applied to a previously activated (5 ml of methanol followed by 5 ml of water) Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). For the determination of rhoifolin, the cartridge was first washed with 2.5 ml of distilled water and then eluted with 5 ml of 80% methanol. For the determination of daidzin, the cartridge was washed with 2.5 ml of 20% methanol and then eluted with 2 ml of 80% methanol. After evaporating the eluate at 70°C under a stream of nitrogen, the residue was dissolved in 2.5 ml of methanol and the solution was filtered through an HLC-Disc filter (Kanto Chemicals, Tokyo, Japan). The filtrate was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 50 μl of the mobile phase. A 20-μl portion of the solution was subjected to HPLC.

2.3. Apparatus and HPLC conditions

HPLC analyses were performed on a Hitachi (Tokyo, Japan) Model 655 liquid chromatograph equipped with a Model 638 variable-wavelength detector. 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. The detection wavelength was set to 333 nm for rhoifolin. The mobile phase was acetonitrile–0.1 *M* ammonium acetate solution (pH 7.1) (20:80, v/v) and the flow-rate was 0.9 ml min^{-1}. Daidzin was detected at 250 nm. The mobile phase was methanol–0.1 *M* ammonium acetate solution (pH 7.0) (33:67) and the flow-rate was 0.7 ml min^{-1}.

2.4. Calibration

To each standards containing known amounts of rhoifolin (28.9, 49.6, 103.3, 144.6, 206.5 and 247.8 ng) or daidzin (28.0, 48.0, 80.0, 120.0 and 200.0 ng) were added 103.5 ng (for rhoifolin) or 144.9 ng (for daidzin) of genistin as an internal standard. Each sample was prepared in duplicate or quadruplicate. After evaporating the solvent to dryness, the residue was dissolved in 50 μl of the mobile phase. A 20-μl portion of the solution was subjected to HPLC. The peak-height ratios (rhoifolin or daidzin to genistin) were determined in duplicate or quadruplicate. The calibration graphs were obtained by a least-squares linear fitting of the peak-height ratios *versus* the amounts of rhoifolin or daidzin.

2.5. Accuracy

Accuracy was determined for rhoifolin and daidzin by assaying six preparations of 1.0-ml aliquots of human plasma containing two different amounts of rhoifolin (82.6 or 165.0 ng) or daidzin (40.0 ng and 100.0 ng) and a fixed amount of genistin as the internal standard (103.5 and 144.9 ng, respectively). After preparing the sample for HPLC as described above, the peak-height ratios (rhoifolin or daidzin to genistin) were measured.

2.6. Sensitivity

Sensitivity was determined by injecting standard solutions containing known amounts (0.5–3.0 ng) of rhoifolin or daidzin into the HPLC system.

3. Results and discussion

HPLC using reversed-phase columns (RP-HPLC) is often a method of choice for the determination of flavonoids [6]. A mobile phase consisting of acetonitrile–water or methanol–water is commonly used for the RP-HPLC of flavonoids. In this study, the HPLC behaviours of the two PMN-activating flavonoids, rhoifolin and daidzin, were examined by using an Inertsil ODS-2 reversed-phase column with solvent systems containing ammonium acetate. The use of ammonium acetate is of interest for the future determination of flavonoids by liquid chromatography–thermospray mass spectrometry.

To accomplish successful determinations of rhoifolin and daidzin in human plasma by HPLC, it is essential that the analytes are completely separated on the chromatogram from interfering material present in the plasma. It has been reported that Sep-Pak C_{18} cartridges provide a simple method for the extraction and clean-up of flavonoids in plant extracts [7–9] and animal or human body fluids [10–12] prior to HPLC or GC.

When methanol was used as the eluent for a Sep-Pak C_{18} cartridge, it was found that the eluate resulted in significant interferences on the chromatogram. This caused difficulties in determining rhoifolin or daidzin at the nanogram level. This problem was overcome, however, by elution of the loaded Sep-Pak C_{18} cartridge with 80% aqueous methanol. Fig. 2 illustrates the chromatograms of (A) an extract of rhoifolin-free plasma and (B) an extract of a plasma sample containing 103.3 ng of rhoifolin and 103.5 ng of genistin. Similarly, chromatograms are presented in Fig. 3 for (A) a daidzin-free plasma sample and (B) a plasma sample spiked with 40.0 ng of daidzin and 144.9 ng of genistin. There was a small shoulder peak present on the rhoifolin signal (Fig. 2B), whereas no interfering compounds were extracted from plasma spiked with daidzin (Fig. 3). The shoulder peak, however, was found not to interfere significantly with the determination of rhoifolin in human plasma at plasma concentrations higher than 15 ng/ml. The recovery of the flavonoids was calculated by

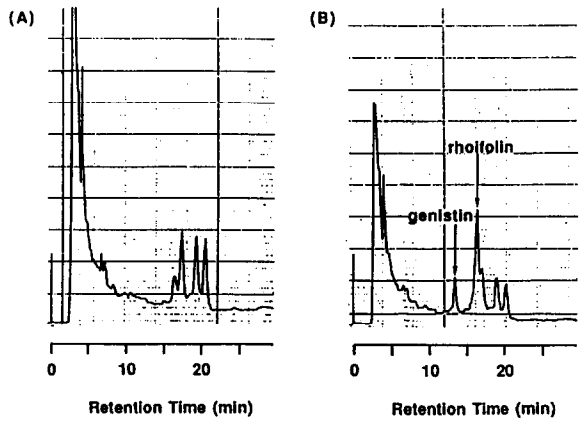

Fig. 2. HPLC of extracts of (A) blank plasma and (B) plasma spiked with rhoifolin (103.3 ng) and genistin (103.5 ng).

comparing the peak heights before and after the Sep-Pak extraction procedure. Elution with 80% aqueous methanol gave recoveries of 82.2% for rhoifolin and 97.2% for daidzin.

Calibration graphs were prepared in the ranges 30–250 ng of rhoifolin and 30–200 ng of daidzin by using genistin as the internal standard. The peak-height ratios were plotted against the mixed mass ratios of rhoifolin or daidzin to genistin. A good correlation was found between the observed peak-height ratios (y) and the mixed mass ratios (x). A least-squares regression analyses gave the regression lines $y = 0.0271x + 0.0276$ ($r = 0.998$) for

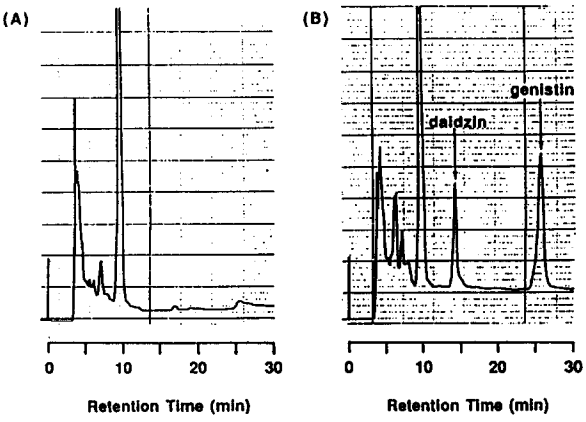

Fig. 3. HPLC of extracts of (A) blank plasma and (B) plasma spiked with daidzin (40.0 ng) and genistin (144.9 ng).

Table 1
Linear regression analyses of calibration plots for rhoifolin and daidzin: peak-height ratios (y) versus mass ratios (x)[a]

Compound	Slope	y-Intercept	r
Rhoifolin[b]	0.0271 ± 0.0008	0.0276 ± 0.0791	0.998 ± 0.000
Daidzin[c]	0.0113 ± 0.0005	−0.0467 ± 0.0161	0.999 ± 0.001

[a] Values are means ± S.D.
[b] From three regression lines.
[c] From four regression lines.

rhoifolin and $y = 0.0113x - 0.0467$ ($r = 0.999$) for daidzin (Table 1).

The accuracy of measurements was determined by adding rhoifolin or daidzin to 1.0-ml aliquots of pooled plasma. To the plasma samples were added fixed amounts of genistin (103.5 ng for rhoifolin and 144.9 ng for daidzin) and different amounts of rhoifolin (82.6 and 165.0 ng) or daidzin (40.0 and 100.0 ng). Table 2 shows that the amounts of rhoifolin or daidzin added were in good agreement with the amounts of rhoifolin or daidzin measured, the relative errors being less than 6% for rhoifolin and 2% for daidzin. The inter-assay relative standard deviations (R.S.D.s) ($n = 6$) were less than 3% for both rhoifolin and daidzin. The sensitivity of the HPLC assay was 2 ng for rhoifolin and 0.5 ng for daidzin on-column with a signal-to-noise ratio of about 3 (Fig. 4).

The present method provides a sensitive and reliable technique for the determination of plasma concentrations of rhoifolin and daidzin. Using genistin as the internal standard for the RP-HPLC assay, good accuracy and precision are obtained.

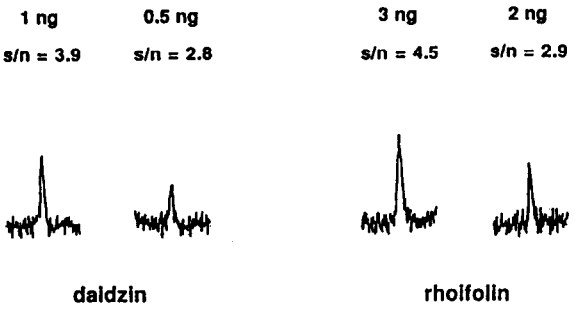

Fig. 4. Sensitivity of the method, s/n = Signal-to-noise ratio.

4. References

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Table 2
Accuracy of HPLC determination of rhoifolin and daidzin in plasma

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values						Mean ± S.D.		
Rhoifolin									
82.6	86.2	88.4	85.8	87.3	86.6	89.0	87.2 ± 1.27	+5.6	1.5
165	170	164	173	171	170	178	171 ± 4.56	+3.6	2.7
Daidzin									
40.0	38.7	39.1	39.7	39.5	39.3	40.5	39.5 ± 0.61	−1.3	1.5
100	104	99.0	99.0	96.5	96.5	102	99.5 ± 3.00	−0.5	3.0

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