A validated HPLC determination of the flavone aglycone diosmetin in human plasma

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ABSTRACT: Diosmetin, 3′,5,7-trihydroxy-4′-methoxyflavone, is the aglycone of the flavonoid glycoside diosmin that occurs naturally in foods of plant origin. Diosmin exhibits antioxidant and anti-inflammatory activities, improves venous tone and it is used for the treatment of chronic venous insufficiency. Diosmin is hydrolyzed by enzymes of intestinal microflora before absorption of its aglycone diosmetin. A specific, sensitive, precise and robust HPLC assay for the determination of diosmetin in human plasma was developed and validated. Diosmetin and the internal standard 7-ethoxycoumarin were isolated from plasma by liquid–liquid extraction and separated on a C8 reversed-phase column with methanol–water–acetic acid (55:43:2, v/v/v) as the mobile phase at 43°C. Peaks were monitored at 344 nm. The method was linear in the 10–300 ng/mL concentration range (r > 0.999). Recovery for diosmetin and internal standard was greater than 89.7 and 86.8%, respectively. Intra-day and inter-day precision for diosmetin ranged from 1.6 to 4.6 and from 2.2 to 5.3%, respectively, and accuracy was better than 97.9%. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: diosmin; diosmetin; antioxidant; HPLC determination; UV detection

INTRODUCTION

Flavonoids are polyphenolic secondary metabolites that are usually occur as glycosides in foods of plant origin, such as vegetables, beverages and citrus fruits (Justesen et al., 1998; Kawai et al., 1999; Wiseman et al., 2001; Kanaze et al., 2003). Flavonoids are classified, according to their chemical structure, into flavones, flavonols, flavanones, isoflavones and anthocyanins (Rice-Evans and Miller, 1996). Dietary flavonoids have been demonstrated to possess a variety of pharmacological effects, such as antioxidant and free radical scavenging (Kandaswami and Middleton, 1994; Anila and Vijayarakshmi, 2003), anti-inflammatory (Middleton and Kandaswami 1992; Rotelli et al., 2003) and anti-ulcer (izzo et al., 1994). Their intake has been found to correlate to a reduced risk of some chronic diseases, such as different types of cancer, asthma and cardiovascular disease (Hertog et al., 1995; Bernatova et al., 2002; Knekt et al., 2002).

Diosmin is the 7-rhamnoglycoside of diosmetin and possesses antioxidant (Bouskela et al., 1997) and anticarcinogenic activities (Tanaka et al., 1997). It enhances venous tone and microcirculation (Taskin et al., 1996; Amiel and Barbe, 1998) and protects capillaries (Lonchampt et al., 1989; Galley and Thiollet, 1993), mainly by reducing systemic oxidative stress (Unlu et al., 2003). Formulations of micronised diosmin and hesperidin (450/50 mg) are used for the treatment of chronic venous insufficiency (Guillot, 1994), hemorrhoids (Bucksehe et al., 1997), venous ulcers, especially of the lower limbs (Guilhou et al., 1997; Struckmann, 1999; Smith, 2003; Roztocil et al., 2003), and the prevention of postoperative thromboembolism (Tsimoyiannis et al., 1996). Diosmin is hydrolyzed by the enzymes of intestinal bacteria into its flavone aglycone, diosmetin, which is subsequently absorbed into the systematic circulation. In contrast to other citrus flavonoid aglycones which are found in plasma mainly as their conjugated forms (Kanaze et al., 2004), diosmetin can be detected in human plasma as its unconjugated form (Cova et al., 1992).

To our knowledge only one HPLC method combined with mass spectrometry detection has been reported for the determination of diosmetin in biological fluids (Cova et al., 1992). Therefore, the objective of the current study was to develop and validate a simple liquid–liquid extraction HPLC-UV method for the quantitative determination of diosmetin in human plasma that is capable of conducting pharmacokinetic studies after intake of foods or oral administration of pharmaceutical preparations that contain diosmin, either alone or in combination with other flavonoid glycosides.
**EXPERIMENTAL**

**Chemicals and reagents.** Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) was purchased from LKT Laboratories (Minnesota, MN, USA). Internal standard, 7-ethoxycoumarin, was obtained from Sigma (St Louis, MO, USA). HPLC-grade methanol, diethyl ether, acetic acid, and dimethyl sulfoxide were supplied from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

**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 x 4.6 mm i.d., 5 µm particle size; Rigas Labs, Thessaloniki, Greece), followed by a guard column (20 x 4.6 mm i.d.) dry packed with pellicular ODS material (37–53 µm; Whatman, Kent, UK). The mobile phase consisted of methanol–water–acetic acid (55:43:2, 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 0.9 mL/min at 43°C and the detector was set at 344 nm. The integrator attenuation was 2 and the chart speed was 0.1 cm/min.

**Standard solutions.** Stock solutions of diosmetin and the internal standard 7-ethoxycoumarin were prepared daily by dissolving appropriate amounts of the compounds in methanol/dimethyl sulfoxide (50/50, v/v) to achieve concentrations of 400 µg/mL for each compound. Appropriate solutions of the stock solution of diosmetin were made to prepare the working solutions containing 0.5, 1.25, 2.5, 5, 10, 12.5 and 15 µg/mL of diosmetin. The stock solution of the internal standard was also diluted with methanol to give the working solution of 25 µg/mL of 7-ethoxycoumarin. Calibration standard samples were freshly prepared in 1 mL of human plasma by spiking control pools with 20 µL of the diosmetin working solutions and 20 µL of the internal standard working solution to yield concentrations corresponding to 10, 25, 50, 100, 200, 250 and 300 ng/mL of diosmetin.

**Quality control samples.** Volumes of 25 mL of human plasma spiked with appropriate amounts of diosmetin stock solutions to obtain quality control samples containing 50, 100 and 200 ng/mL of diosmetin. The samples were divided into aliquots of about 2 mL into one-dram vials capped tightly and placed at −20°C, pending analysis. These samples were used in the analysis of plasma samples as quality controls for the purpose of checking recovery of diosmetin in the daily analyses of plasma samples.

**Sample preparation.** Plasma samples (1 mL) were spiked with diosmetin and the internal standard. Then they were diluted with 2 mL of phosphate buffer (0.1 M, pH 2.4). Liquid–liquid extraction of diosmetin and internal standard was carried out by the addition of 6 mL of diethyl ether, followed by shaking for 30 min at 37°C. After centrifugation at 3000 rpm for 30 min, the samples were kept at −20°C for 30 min and then the upper organic phase was decanted and evaporated to dryness at 25°C with the aid of a gentle stream of dry air. Finally, the residue was dissolved in 100 µL of mobile phase and a 20 µL aliquot was injected into the chromatographic system for quantification.

**RESULTS AND DISCUSSION**

**Optimization of separation conditions**

The chromatographic conditions described in this assay were arrived after investigating C18 and C8 columns and different mobile phases consisting of aqueous mixtures of methanol or acetonitrile in different ratios. The better chromatographic separation of the analytes was achieved using a C8 reversed-phase column with methanol–water–acetic acid (55:43:2, v/v/v) as the mobile phase. The addition of acetic acid in the mobile phase, which suppresses the ionization of phenolic groups, as well as the column temperature (43°C), was found to further improve the separation of the analytes and the symmetry of the chromatographic peaks.

Figure 1 illustrates typical chromatograms obtained with a blank plasma and a plasma sample containing 61.2 ng/mL of diosmetin obtained from a female.
Accuracy and precision

Intra-day precision was determined by calculating the %RSD for six determinations at each concentration of three quality control samples (50, 100 and 200 ng/mL) and was found to be less than 4.6%. Intra-day accuracy, assessed by calculating the estimated concentrations as a percentage of the nominal concentrations, was better than 98.7% (Table 1).

Inter-day precision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions. Inter-day precision for diosmetin was 5.3% based on %RSD values of 5.3, 3.3 and 2.2% for quality control samples containing 50, 100 and 200 ng/mL. Inter-day accuracy for diosmetin was found to be 98.0, 101.4 and 97.9% for samples containing 50, 100 and 200 ng/mL (Table 1).

Limit of quantification

The limit of quantification, defined as the lowest quantifiable concentration on the calibration curve at which both accuracy and precision should be within the maximum tolerable CV of 15%, was deemed to be 10 ng/mL of diosmetin, using a 1 mL plasma sample. The limit of quantification is comparable to that reported previously (Cova et al., 1992).

Robustness

Robustness is the capability of an analytical procedure to remain unaffected by small but deliberate variations in the method parameters (Jimidar et al., 1998). The robustness of the method was performed by evaluating small variations in mobile phase composition (±2%) and column temperature (±3°C). By increasing the organic phase content in mobile phase and the column temperature resulted in a decrease of the retention time of both diosmetin and internal standard. However, the concentration of the quality control samples was not significantly affected by these small changes during sample processing.
(Tables 2 and 3). Therefore, the method is robust with respect to small changes in mobile phase composition and column temperature. Furthermore, the selectivity of the method over time was not significantly affected and more than 800 sample injections were performed without loss of column performance.

CONCLUSION

This study led to the development and validation of a liquid–liquid extraction HPLC-UV method for the quantitative determination of the flavone aglycone diosmetin in human plasma. The method was simple, inexpensive, specific, sensitive, precise, accurate and robust and seems suitable for the conduction of pharmacokinetic studies in humans after the intake of foods of plant origin or pharmaceutical formulations that contain the flavone glycoside diosmin.

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