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Quantitation of phlorizin and phloretin using an ultra high performance liquid chromatography–electrospray ionization tandem mass spectrometric method

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ABSTRACT

A sensitive and selective ultra high performance liquid chromatography–tandem mass spectrometric (UHPLC–MS/MS) method for the determination of phlorizin and phloretin in human plasma has been firstly developed. Samples were prepared after protein precipitation and analyzed on a C_{18} column interfaced with a triple quadrupole tandem mass spectrometer. Negative electrospray ionization was employed as the ionization source. The mobile phase consisted of acetonitrile–water (0.02% formic acid), using a gradient procedure. The analytes and internal standard dihydroquercetin were both detected by use of multiple reaction monitoring mode. The method was linear in the concentration range of 2.5–1000.0 ng/mL. The lower limit of quantification (LLOQ) was 2.5 ng/mL. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 9.2%. The accuracy determined at three concentrations was within ±7.3% in terms of relative error. The total run time was 12.0 min. This assay offers advantages in terms of expediency, and suitability for the analysis of phlorizin and phloretin in various biological fluids.

1. Introduction

Phenolic compound phloretin and its glycosylated product phloretin-2-b-D-glucose (phlorizin; also called phloridzin, phlorhizin, phlorhizin or phlorizoside) belong to the chemical class of dihydrochalcones, phenylpropanoids with structures closely related to those of the immediate flavonoid precursors, the chalcones [1]. They consist of a C6–C3–C6 skeleton structure (Fig. 1). As the principal flavonoids, they are mainly found in apples and processed apple products such as apple juice, which are frequently consumed by humans [2]. Due to their various biological functions, including antioxidase activity, block glucose absorption, and the inhibition of the expression of *interleukin 8* (IL-8), C-X-C motif chemokine ligand 10 (CXCL10), and tumor necrosis factor alpha (TNF-a) mRNAs in lipopolysaccharide (LPS)-stimulated human acute monocytic leukemia-derived cell line, phloretin and phlorizin have received frequent usage in fields of foods, beverages, food additives, pharmaceuticals as well as cosmetics [3,4]. Hundreds' research articles in relation to various pharmacological effects of phloretin and phlorizin have been published [5–7]. In contrast, knowledge about their pharmacokinetics is limited. It is critical to learn absorption, distribution, metabolism, and excretion of phloretin and phlorizin since polyphenolic compounds of apple may play an important role in physiologic functions related to human health.

A sensitive and user friendly analytical method to determine the concentration of phloretin and phlorizin in biological fluids is necessary for the timely and accurate translation of *in vitro* findings to the clinical setting. So far, there is no well-established method to determine the phlorizin in biological samples. These considerations underscore the potential utility of a rapid and convenient assay for the measurement of phloretin and phlorizin. Several high-performance liquid chromatography methods have been developed for the determination and identification of phloretin and phlorizin. For example, Lommen et al. identified and confirmed the presence of phlorizin in the apple peel by directly coupled HPLC–NMR-MS [8]. Tsao et al. determined phlorizin in eight apple cultivars using liquid chromatography with

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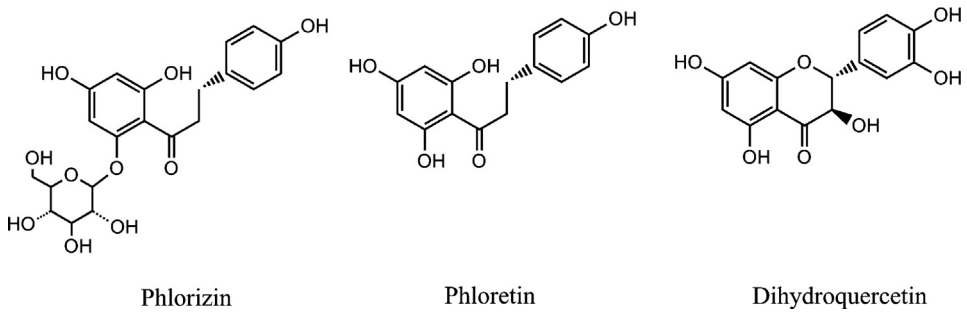

Fig. 1. The structures of phlorizin and phloretin.

a diode array detector [9]. Crespy et al. reported a method for analysis of phloretin and phlorizin in rat plasma and urine using HPLC with a multielectrode coulometric detection [10]. But the necessary of coulometric detector was a restriction in terms of cost, time-consuming and general applicability. In addition coulometric detector was able to interfere with endogenous plasma constituents or mobile phase.

In this paper, we described a simpler, selective and highly sensitive UHPLC–MS/MS method using small volumes of plasma from humans, which allow the simultaneous quantification of phloretin and phlorizin based on a simpler protein precipitation extraction. This method is fully validated for clinical studies and therefore could be the basis for further clinical studies with phloretin and phlorizin.

2. Experimental

2.1. Materials

Phloretin, phlorizin and dihydroquercetin (internal standard, I.S.) were purchased from Sigma (St. Louis, MO). HPLC-mass grade methanol, acetonitrile and formic acid were purchased from Anaqua Chemical Supply (Houston, TX). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA).

2.2. Instrumentation

The chromatographic system used consisted of an Agilent 1290 infinity UPLC system, including 1290 Infinity Binary Pump with integrated vacuum degasser (G4220A), thermostat (G1330B), standard autosampler (Model G4226A) and thermostated Column Compartment (Model G1316C). The mass spectrometer was an Agilent 6490 mass spectrometer equipped with iFunnel technology (Agilent, Singapore). (Ion Funnels for the Masses: Experiments and Simulations with a Simplified Ion Funnel) Data acquisition was carried out by Agilent mass Hunter® workstation B.05.00 software on a DELL computer.

2.3. LC–MS conditions

The chromatographic separation was achieved on a ZORBAX extend-C_{18} column (100 mm × 2.1 mm i.d., 1.8 μm, Agilent, Palo Alto, CA). The mobile phase flow-rate was set at 0.3 mL/min, and the column temperature was maintained at 35 °C. The mobile phase consisted of a linear gradient of 0.02% (v/v) formic acid (A) and acetonitrile (B): 0–4.0 min, 5–20%B (v/v); 4.0–6.5 min, 20–40%B; 6.5–6.6 min, 40–90%B; 6.6–7.6 min, 90–90%B; 7.6–8.0 min, 90–5%B; 8.0–12 min, 5–5%B.

The mass spectrometer was equipped with an electrospray ionization source (ESI) operating in negative mode quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 273.0 → m/z 167.1for phloretin, m/z 435.1 → m/z 273.1 for phlorizin, m/z 301.0 → m/z 151.0 for dihydroquercetin (internal standard, I.S.), respectively, with a dwell time of 100 ms.

In order to optimize all the MS parameters, a standard solution (1 μg/mL) of the analytes and I.S. was infused into the mass spectrometer. Some mass spectrometer parameters were identical for all analytes. Source conditions included drying gas at 250 °C, sheath gas at 300 °C, and 15 L/min flow for drying gas and 11 L/min flow for sheath gases, respectively. The ion spray voltage was set at 3500 V. The nebulizer gas reached 35 psi. The funnel exit DC voltage was set at 50 V. The funnel voltage drop was set at 200 for high pressure funnel and 100 for low pressure funnel, respectively. The RE voltage was 150 for high pressure funnel and 100 for low pressure funnel. The collision energy (CE) and cell accelerate voltage (CAV) were optimized for each analyte. The values of the collision energy were 10, 14 and 18 V for phlorizin, phloretin and I.S., respectively. The cells accelerate voltage were 3 for all of them.

2.4. Sample preparation

The plasma was prepared by removing protein through a precipitation method. In a 1.5 mL centrifuge tube an aliquot 100 μL of human plasma was spiked with 10 μL of dihydroquercetin solution (internal standard, 1 μg/mL). After vortexing, 400 μL of methanol was added to the tubes and the tubes were vortex mixed for 3 min. After centrifugation at 14,000 rpm for 15 min in a refrigerated microcentrifuge (Labnet International, Woodbridge, NJ) at 4 °C, 5 μL of the clear supernatant fluid was injected into the UHPLC–MS/MS system for phloretin and phlorizin. The prepared samples were kept in an autosampler at 4 °C until injection.

2.5. Preparation of standard and quality control samples

Stock solutions of phloretin and phlorizin were prepared in methanol at the concentration of 1000 μg/mL. Stock solution of I.S. was prepared in methanol at the concentration of 500 μg/mL and diluted to 1 μg/mL with methanol. Calibration curves were prepared by spiking the appropriate standard solution in 0.1 mL of blank plasma. Effective concentrations in plasma samples were 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/mL. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 10, 100 and 500 ng/mL f. The spiked plasma samples (standards and quality controls) were then treated following the "sample preparation" procedure on each analytical batch along with the unknown samples.

2.6. Method validation

Plasma samples were quantified using the ratio of the peak area of each analytes to that of I.S. as the assay parameter. Peak area ratios were plotted against analytes concentrations and standard curves were in the form of $y = A + Bx$.