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Journal of Chromatography B, 821 (2005) 194–201
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–tandem mass spectroscopy assay for quercetin and conjugated quercetin metabolites in human plasma and urine

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Received 4 March 2005; accepted 2 May 2005

Abstract

A sensitive and specific method was developed and validated for the quantitation of quercetin in human plasma and urine. The application of liquid chromatography–tandem mass spectrometry (LC/MS/MS) with a TurboIonspray (TIS) interface in negative mode under multiple reactions monitoring was investigated. Chromatographic separation was achieved on a C_{12} column using a mobile phase of acetonitrile/water with 0.2% formic acid (pH 2.4) (40/60, v/v). The detection limit was 100 pg/ml and the lower limit of quantification was 500 pg/ml for plasma samples; the detection limit was 500 pg/ml and the lower limit of quantification was 1 ng/ml for urine samples. The calibration curve was linear from 1 to 800 ng/ml for plasma samples and was linear from 1 to 200 and 50 to 2000 ng/ml for urine samples. All the intra- and inter-day coefficients of variation were less than 11% and intra- and inter-day accuracies were within ±15% of the known concentrations. This represents a LC/MS/MS assay with the sensitivity and specificity necessary to determine quercetin in human plasma and urine. This assay was used to determine both parent quercetin and the quercetin after enzymatic hydrolysis with β-glucuronidase/sulfatase in human plasma and urine samples following the ingestion of quercetin 500 mg capsules.

Keywords: LC/MS/MS; Quercetin; Flavonoid; Analysis

1. Introduction

Flavonoids are phenolic compounds widely present in plants and foods of plant origin and they comprise one of the largest and most widely distributed groups of secondary plant metabolites [1]. Quercetin (Fig. 1) represents one of the most abundant flavonoid in foods [2], being present in oranges, onions, apples, wine and tea in the form of glycosides. It exhibits a variety of biological activities, including cardiovascular protection and anti-cancer, anti-inflammatory and antioxidant activities [1–3]. It is also ingested as a component of many herbal products and dietary supplements including Quercetin Plus®, Quercetin Complex® and Natrol Quercetin®.

Numerous analytical methods have been reported for the quantitation of total quercetin concentrations in biological samples, obtained after the enzymatic hydrolysis of conjugated quercetin metabolites, and for the analysis of quercetin metabolites. Analytical methods have included LC/MS [4,5], LC/MS/MS [6–8], HPLC with UV detection [9], HPLC with fluorescent detection [10], HPLC with electrochemical detection [7,10–14] and HPLC-radiocounting and tandem mass spectrometry [11]. Following onion ingestion, Witting et al. [6] attempted to analyze unchanged quercetin, but were only able to detect quercetin conjugates while Mullen et al. [7] reported trace levels of unchanged quercetin in one subject. Jin et al. [12] have reported a very sensitive assay for quercetin using electrochemical detection which they used to determine total quercetin, following enzymatic hydrolysis, in human plasma after green tea ingestion. Unchanged quercetin has not been determined in human plasma; therefore, knowledge

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doi:10.1016/j.jchromb.2005.05.009

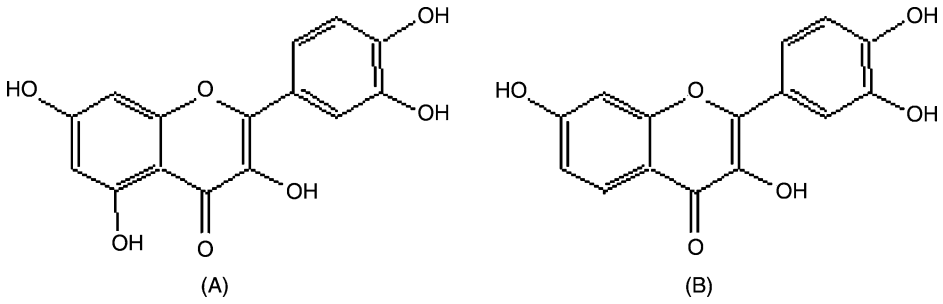

Fig. 1. Chemical structures of quercetin (A) and the internal standard fisetin (B).

about the bioavailability and pharmacokinetic characteristics of quercetin in humans is limited.

We developed and validated a sensitive and specific method to measure both the parent and conjugated quercetin in human plasma and urine using LC/MS/MS. Conjugated quercetin metabolites were analyzed following the enzymatic hydrolysis of quercetin conjugates in plasma and urine using β-glucuronidase and sulfatase. This new analytical method allows the analysis of low concentrations of parent quercetin in human plasma and urine with good reproducibility.

2. Experimental

2.1. Materials and methods

Quercetin dihydrate (99%), fisetin (99%), formic acid (98%), β-glucuronidase (Type B-10, from bovine liver 10,400 units/mg solid), sulfatase (Type VI, 4.9 mg/ml, 3.9 units/mg prot.), formic acid, acetic acid and trifluoroacetic acid were all obtained from Sigma–Aldrich (St. Louis, MO). Methanol, acetonitrile and water were all HPLC grade and purchased from Fisher Scientific (Springfield, NJ). Quercetin 500-Plus® capsules were purchased from Herbal Fields Supplements (Jacksonville, FL).

2.2. Preparation of standard solutions for plasma and urine samples

A stock solution of 1 mg/ml quercetin (Fig. 1A) was prepared in methanol. Dilution of the stock solution with methanol yielded working stock solutions at concentrations of 0.02, 0.04, 0.08, 0.4, 1.6, 4.0, 8.0, 12 and 16 μg/ml for plasma. For urine two standard curves were constructed over the range of 1–200 ng/ml and over the range of 50–2000 ng/ml. Working stock solutions of concentrations of 0.02, 0.08, 0.4, 1.0, 1.6, 2.4, 3.2 and 4 μg/ml and of concentrations of 1, 2, 4, 8, 12, 16, 20, 30 and 40 μg/ml were prepared for these two standard curves, respectively. A stock solution of the internal standard fisetin (Fig. 1B) was prepared in methanol at a concentration of 1 mg/ml and diluted with methanol to produce a solution with a concentration of 4 μg/ml. A 20 μl aliquot of quercetin stock solution, 20 μl fisetin stock solution and 20 μl formic acid were added to 360 μl blank plasma or urine and vortexed for 1 min prior to extraction.

For plasma samples, standard solutions for precision and accuracy determinations were prepared at quercetin concentrations of 1, 10, 100 and 600 ng/ml in methanol. For urine samples, standard solutions for precision and accuracy determinations were prepared at quercetin concentrations of 4, 100 and 200 ng/ml in methanol.

2.3. Sample extraction

An aliquot of 380 μl of human plasma or urine was transferred into a 2 ml eppendorf tube and 20 μl of a 4 μg/ml solution of fisetin (final concentration of 200 ng/ml) and 20 μl formic acid added. The solution was vortexed for 20 s. Acetone (800 μl) was added to precipitate plasma proteins; the samples were vortexed for 1 min and centrifuged at 20,800 × *g* for 30 min. One milliliter of the supernatant was aspirated into a 5 ml glass tube, dried under a N_2 stream and reconstituted with 200 μl mobile phase by vortex mixing. The reconstituted sample was transferred into a 200 μl autosampler vial insert for analysis by LC/MS/MS.

2.4. Enzymatic hydrolysis of conjugated metabolites

The pH of the urine samples (25 μl) was adjusted to pH 5 by the addition of 75 μl of 2 M sodium acetate buffer containing 10 mg/ml ascorbic acid. Argon gas was layered over the sample, followed by the addition of glucuronidase (5 μl, 50 U/μl) and sulfatase (8 μl, 19.11 unit/ml) for human plasma or glucuronidase (10 μl, 50 U/μl) and sulfatase (20 μl, 19.11 unit/ml) for human urine, along with the internal standard, fisetin (10 μl, 2 μg/ml). The samples were incubated in a sealed vial for 1 h at 37 °C with continuous shaking. After hydrolysis, 1 ml of ice-cold methanol was added to the sample and it was evaporated to dryness using nitrogen gas. Samples were reconstituted with 100 μl of mobile phase and vortexed for 2 min, then centrifuged at 20,800 × *g* for 5 min. Following this, 20 μl of the supernatant was used for injection.