The Quantitation of Metabolites of Quercetin Flavonols in Human Urine

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Abstract

The flavonoid quercetin, or its metabolites, inhibit chemical carcinogenesis in rodents and may have a role in the prevention of human cancers. Quercetin exposure in human populations results from the dietary intake of various plant foods; high concentrations of quercetin are found in apples, onions, tea, and red wine. Determination of the relationship between dietary intake and cancer risk depends on the characterization of quercetin intake. The development and use of biomarkers for quercetin intake may provide a basis for the objective classification of this exposure. One possible biomarker is metabolic products of quercetin. We report the development of a high-performance liquid chromatography (HPLC)-based assay for quantitation of quercetin metabolites in human urine. The metabolites include 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid), metahydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid). The assay has only two major steps, ether extraction and HPLC analysis, and is suitable for analysis of large sample numbers. Analytical characteristics of the assay include a sensitivity of less than 1 μg, precision with coefficients of variation <10%, and metabolite recoveries >90%. The mean concentrations of 3,4-dihydroxyphenylacetic acid, metahydroxyphenylacetic acid, and homovanillic acid in two human urine samples are approximately 0.7, 4.8, and 2.8 μg/ml, respectively. The identification of each metabolite is confirmed by HPLC, UV absorbance scans, and gas chromatography-mass spectrometry analysis. These results verify the occurrence of quercetin metabolites in human urine and the feasibility of quercetin metabolite quantitation, by the assay described herein, for epidemiological studies. Development of the analytical procedure is an essential first step for validation of the metabolites as biomarkers of quercetin intake.

Introduction

Human diets contain a wide range of compounds with possible "semi-essential" functions. One group of such compounds is the flavonoids. Flavonoids have multiple chemical and biological actions, including antioxidant (1-4), chelation (5, 6), anticarcinogenic (7, 8), bacteriostatic (9), and secretory activities (10, 11). Several enzymes, including trypsin, leucine aminotransferase, phosphosulfotransferase, DNA topoisomerase, phosphoinositol kinase, NADPH diaphorase, H⁺,K⁺-ATPase, and xanthine oxidase, are modulated by flavonoids (12-18). Furthermore, some flavonoids may act as antiviral, antitumor, anti-inflammatory and antiallergenic agents (19-21).

Flavonoids have a widespread distribution among food plants. In general, fruits and vegetables are good dietary sources of flavonoids (22-28). The flavonoids are found primarily in the outer layers of fruits and vegetables; the peel and outer leaves have the highest concentrations (22, 23, 29-32). Human consumption of flavonoids is estimated at 1 g or more per day for the average American diet (33).

One flavonoid, quercetin, is of particular interest because of its anticarcinogenic activities (7) and a significant quantitative presence in human foods. Quercetin is found in numerous fruits and vegetables (22-24), the highest concentrations occurring in apples, onions, and tea (34-36). Biological activities of quercetin include the inhibition of cell proliferation in animals and cell cultures (37) and prevention of chemically induced tumors. Early reports of carcinogenic activity by quercetin are based on bacterial mutagenicity tests (38). Extrapolation of the results to humans, in this case, led to an erroneous conclusion. Tests of the carcinogenicity of quercetin in mammalian species, with few exceptions (39, 40), have proven to be negative (41, 42), and epidemiological data do not implicate carcinogenic activity for quercetin in humans (43).

Interpretations of more recent data from in vitro experiments, suggesting carcinogenic activity for quercetin, do not consider the absence of quercetin absorption (44) and almost complete metabolism of quercetin in the human gastrointestinal tract (45). Thus, the evidence to date does not support carcinogenic activities for quercetin in humans (46, 47) but rather a chemopreventive effect. The chemopreventive effect may be mediated by metabolites of quercetin. Also, quercetin or its metabolites may have cytotoxic activities (49).

Because diets associated with a reduction in cancer risk generally contain numerous potential anticarcinogens (43, 50, 51), biomarkers of dietary quercetin would be useful in studies of diet-cancer relationships. Studies on the metabolism of quercetin suggest degradation by intestinal microbes and relatively low absorption of quercetin (52, 53), limiting the use of quercetin as a biomarker. However, metabolites of quercetin have
Fig. 1. Chromatograms of standards and urine sample extracts. A, chromatogram from the analysis of a mixture containing 5 μg of each standard, prepared from crystalline compounds. B and C, chromatograms from the analysis of urine sample extracts. The extracts yielding the chromatograms shown in B and C came from separate urine sample collections by two individuals.
potential as biomarkers of quercetin and quercetin-containing food intake. Metabolism of quercetin yields DHPAA, mHPAA, and HVA (54). These three metabolites are excreted in the urine of rats, rabbits, and humans (54–56).

A prerequisite for the evaluation of metabolic products from quercetin as biomarkers is the development of suitable analytic methods. Presently available methods are inadequate for the identification and quantitation of quercetin metabolites (54, 57–60). We report the development of a quantitative method for the detection of quercetin metabolites in human urine samples. The method is an HPLC-based assay, using an on-line photodiode detector.

Materials and Methods

Materials. DHPAA, mHPAA, HVA, and p-coumaric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl ether anhydrous was obtained from Mallinckrodt Specialty (Paris, KY). HPLC-grade TFA and BSTFA with 1% trimethylchlorosilane were from Pierce Chemical Co. (Rockford, IL). Acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ).

Preparation of Standard Solutions and Determination of Calibration Curves. Extinction coefficients for DHPAA, mHPAA, HVA, and p-coumaric acid were determined as follows: (a) each crystalline compound was dried to a constant weight at 90°C and solubilized with methanol; (b) the λ maximum was determined for each compound; and (c) absorbance measurements at the λ maxima were determined for DHPAA, mHPAA, HVA, and p-coumaric acid solutions, at known concentrations by weight. The λ maxima and extinction coefficient (1%, 1 cm), respectively, for the compounds were as follows: 272 nm and 172 (DHPAA), 272 nm and 127 (mHPAA), 272 nm and 174 (HVA), and 303 nm and 1176 (p-coumaric acid). Response factors were determined for each analyte, using coumaric acid as an internal standard.

The routine preparation of DHPAA, HVA, mHPAA, and p-coumaric acid standard solutions was done by solubilization of crystalline compounds in methanol, filtration, measurement of absorbance at the respective λ maxima and calculation of concentrations, using the respective compound’s extinction coefficient. Each standard solution was evaluated for purity by HPLC analysis, as described in “HPLC Analysis” below, and all standards were greater than 97% pure. All standard solutions were stable for at least 6 months with storage at −70°C.
Measurement of Quercetin Metabolites

Collection of Urine Samples. Two male volunteers collected 24-h urine samples for 3 consecutive days. The subjects, ages 24 and 40, consumed their habitual diets and maintained their usual light exercise schedule during the collection period. Urine was collected in 1.0-liter polypropylene bottles containing 10.0 ml of 5% acetic acid. Acetic acid was added to retard bacteria growth and stabilize the metabolites of quercetin. Samples were stored in a refrigerator at approximately 4°C until delivered to the laboratory, generally 15 h after the completion of each 24-h collection. Three-day urine pools were prepared for each individual from frozen 24-h collections and stored at -70°C. Collections and pools were prepared twice from each individual. These investigations were done according to the principles of the Declaration of Helsinki (61), which describes ethical principles and recommendations regarding the orientation of research with human beings.

Extraction of DHPAA, mHPAA, and HVA. Twenty-ml aliquots of urine were adjusted with 5.0 normal hydrochloric acid to pH 1.5 and saturated with NaCl. Fifty μg of p-coumaric acid in methanol (30 μl) were added as an internal standard, and each sample was extracted with 20 ml of anhydrous ethyl ether. After initial mixing and venting, the sample was mixed continuously for 10 min on a reciprocal shaker at medium speed (IKA-Works, Cincinnati, OH). The aqueous layer was collected, after low-speed centrifugation (1650 rpm), for phase separation, and re-extracted with 20 ml of anhydrous ethyl ether; the resulting ether extract was combined with the initial ether extract. The combined extracts were evaporated under nitrogen and reconstituted with methanol/0.1% TFA (50 μl), followed by 950 μl of H2O-0.1% TFA. The reconstituted samples were centrifuged at 12,000 rpm in a Beckman microfuge (Fullerton, CA) for 5 min and passed through a 0.45-μm, organic filter from Millipore (Milford, MA). HPLC analysis was performed using 100 μl of the resulting solution. All procedures were done at room temperature. Notably, the combined ether extracts could be stored overnight at 4°C, without any effect on analyte concentration.

HPLC Analysis. The HPLC system consisted of a Vydak C18 column (Hesperia, CA) with a Chrom Tech C2 guard column (Apple Valley, MN), Beckman 507 autosampler (Fullerton, CA), Beckman 116 programmable solvent module, and a Waters 470 scanning fluorescence detector (Millipore, Milford, MA). Both the diode array and fluorescence detectors had scanning capabilities. A chromatogram was developed at a 1.0 ml/min flow rate with a gradient consisting of 0.1% TFA from 0 to 30 min, 0.1% TFA and 10% methanol-0.1% TFA from 30-35 min in a linear gradient, and 0.1% TFA-10% methanol and 0.1% TFA-20% methanol from 35–65 min in a linear gradient. Analyte detection was by absorbance at 280 nm and fluorescence at 310 nm with an excitation wavelength of 280 nm. In addition, absorbance and fluorescence scans were done for analyte identification and verification of standard compound spectrometric properties. The column was washed between each analysis with 40% methanol/20%–0.1% TFA/40% acetonitrile for approximately 10 min.

Mass Spectrometric Analysis. Mass spectra were acquired with a Finnigan MAT (San Jose, CA); the MAT 95 mass spectrometer was equipped with a Hewlett Packard HP 5890 Series II gas chromatograph (Avondale, PA). The ion source temperature was 200°C. An electron energy of 70 eV was used for acquiring electron ionization GC-mass spectrometry data. Using a splitless injector maintained at 250°C, samples were introduced into the ion source via a DB-5 fused silica capillary.

Fig. 2. UV/visible scans of standards and comigrating compounds from urine sample extracts. Scans were taken of standard and comigrating compounds analyzed in chromatograms in Fig. 1, A and B. A, overlays of DHPAA (Fig. 1A) and the corresponding comigrating compound (Fig. 1B). B and C, similar overlays for mHPAA and HVA, respectively.
Fig. 3. (Continues on next page.) Mass-spectrometric analysis of standards and comigrating compounds from urine sample extracts. Spectra are for DHPAA (A) and the corresponding comigrating compound (D), mHPAA (B) and the corresponding comigrating compound (E) and 3) HVA (C) and the corresponding comigrating compound (F).
Fig. 3. Continued.
Results

A mixture of DHPAA, mHPAA, HVA, and p-coumaric acid (internal standard) was analyzed by HPLC analysis. Each analyte produced sharp, symmetrical peaks with absorbances greater than 0.1 units for 5-μg samples (Fig. 1A). The retention times of DHPAA, mHPAA, HVA, and p-coumaric acid were approximately 26.0, 43.0, 48.0, and 58.0 min, respectively.

Fig. 1. B and C, shows chromatograms from the analysis of urine samples from two individuals. Each urine sample extract contained compounds with retention times, relative to the internal standard, identical to those of standards for DHPAA, mHPAA, and HVA. A comparison of chromatograms in Fig. 1, B and C, with each other and the chromatogram in Fig. 1A illustrate important considerations for appropriate application of this assay. (a) The analysis for chromatograms in Fig. 1, A and C, were done on different days; analysis for chromatograms on Fig. 1, A and B, were done on the same day. Slight shifts in absolute retention times of the analytes were seen between analysis days; compare Fig. 1, A and B, with Fig. 1C. Thus, automated data analysis procedures required the use of relative retention times. (b) Significant differences were found in the complexity of chromatograms for samples from the different individuals; compare Fig. 1, B and C. The chromatogram in Fig. 1B was more complex than that in Fig. 1C. Based on the analysis of numerous samples (>100; data not shown), chromatogram C represents a typical chromatogram; the more complex chromatograms were relatively rare (<5%). However, verification of analyte identity by UV/visible absorbance scans was essential for the analysis of the more complex chromatograms. UV/visible scans of absorbance peaks comigrating with standard compounds had spectra identical to the standards (see below). In contrast, surrounding absorbance peaks had spectra different from those of the standard compounds. (c) The retention time of the internal standard, p-coumaric acid, was not affected by the sample matrix (Fig. 1B); it comigrated with the standard (Fig. 1A). In addition, p-coumaric acid migrated in an area of the chromatogram without other peaks as determined by the analysis of a urine sample lacking added p-coumaric acid (data not shown).

Significant amounts of each analyte were present in each urine sample; estimated amounts of DHPAA, mHPAA, and HVA were 0.87, 4.46 and 3.36 μg/ml, respectively (Fig. 1B) and 0.44, 5.09, and 2.30 μg/ml, respectively (Fig. 1C).

Verification of DHPAA, mHPAA, and HVA identification in urine samples was done by several methods. UV/visible scans of DHPAA, mHPAA, and HVA from the chromatographic analysis shown in Fig. 1A and comigrating analytes from the chromatogram shown on Fig. 1B are shown in Fig. 2A (DHPAA), B (mHPAA), and C (HVA). Overlays of the scans indicated identical spectral properties for the standards and comigrating compounds. Slight differences at the ends of absorbance spectra resulted from the normalization of scans for concentration differences between standards and urine extract analytes. The maxima for the standard and urinary compound was identical for each analyte. It was 272 nm for DHPAA, mHPAA, and HVA.

Additional evaluation of DHPAA, mHPAA, and HVA identification in urine samples was done by the analysis of urine samples after the addition of DHPAA, mHPAA, and HVA from standard solutions of these compounds. A comparison of chromatograms from analysis of the original urine sample (no added compounds) and the samples with added DHPAA, mHPAA, or HVA indicated an increase in the respective peaks identified as...
DHPAA, mHPAA, and HVA in the original urine sample (data not shown).

DHPAA, mHPAA, and HVA were derivatized with BSTFA containing 1% trimethylchlorosilane, and each compound was analyzed by GC-mass spectrometry. Full-scan mass spectra from the analysis are shown in Fig. 3 A–C. An identical analysis was done on compounds isolated from urinary extracts that comigrated with DHPAA, mHPAA, and HVA in HPLC analysis. The mass spectra for these analyses are shown in Fig. 3 D–F. DHPAA and the HPLC-comigrating compound from urinary extracts had retention times of 22.49 and 22.44 min, respectively, in GC analysis. The mass spectra for these analytes is shown on Fig. 3, A and D, respectively. The molecular ion found for DHPAA (m/z = 384.1) was in good agreement with the theoretical molecular ion (m/z = 384.47) for the compound. The molecular ion (m/z = 384.1) and all major fragmentation ions from analysis of the HPLC-comigrating compound were in good agreement with those found for DHPAA. Differences in ion intensities were a result of sample amounts used in the analysis.

mHPAA and the HPLC-comigrating compound from urinary extracts had retention times of 17.46 and 17.43 min, respectively, in GC analysis. The molecular ion of mHPAA (m/z = 296.1) was in good agreement with the predicted molecular ion for the compound (m/z = 296.32). The molecular ion (m/z = 296.1) and all major fragmentation ions from analysis of the HPLC-comigrating compound were in good agreement with those found for mHPAA (Fig. 3, B and E). Good agreement was found between the retention times in GC analysis of HVA and the HPLC-comigrating compound (21.29 versus 21.27 min). The molecular ions for the HPLC-comigrating compound and HVA and fragmentation patterns were in good agreement. The predicted, HVA, and HPLC-comigrating compound molecular ions were 326.39, 326.1 and 326.1 m/z, respectively. In summary, the results from HPLC, UV/visible scans and mass spectral analysis indicated that human urine samples contained DHPAA, HPAA, and HVA.

Analytical characteristics of the HPLC-based assay were evaluated for the quantitation of DHPAA, mHPAA, and HVA. Dose-response curves for DHPAA, mHPAA, and HVA are shown on Fig. 4A, B, and C, respectively. Less than 0.25 μg of each analyte/100-μl injection was detectable, and dose-response curves were linear. The lowest r² value from linear regression analysis of the dose-response curves was 0.996.

Precision of the assay was determined by replicate analysis of a second set of urine pools from the two volunteers. Multiple analyses of each urine pool were done on each of 4 days. One urine pool contained mHPAA and HVA; it did not contain DHPAA. The other urine pool contained each of the three analytes. CVs for the analysis are shown in Table 1. The overall CVs were less than 10% for all analytes in either pool. Between-day CVs were slightly higher than within-day CVs for all analytes. Similar results were obtained from the analysis of either pool. Also, replicate analysis indicated that peak height was more precise than peak area for the quantitation of metabolites (data not shown).

### Table 1 Assay precision CVs (%)

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<th>Pool</th>
<th>DHPAA</th>
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<th>HVA</th>
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<td></td>
<td>Within day</td>
<td>Between day</td>
<td>Overall</td>
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### Table 2 Extraction efficiency

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<th>Number of extractions</th>
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<tr>
<td>2</td>
<td>90.5</td>
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<tr>
<td>3</td>
<td>94.5</td>
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Discussion

This article reports the development of an HPLC-based method for the detection and quantitation of DHPAA, mHPAA, and HVA in human urine samples. The method consists of two major steps: ether extraction at acid pH and analysis of the extract by HPLC. These two steps are sufficient and necessary for the detection and quantitation of DHPAA, mHPAA, and HVA in human urine samples. The assay procedure allows for the quantitation of each and all analytes in a single HPLC analysis. Dose-response curves for standards and samples are steep and linear. Assay sensitivity is high and sufficient for the analysis of human urine samples. Approximately 0.25 μg of each analyte can be detected by the assay. High recoveries (>90%) are found in routine analysis. Precision of the assay is high, with an overall coefficient of variation of less than 10% for each analyte. These analytic characteristics are appropriate for the routine detection of DHPAA, mHPAA, and HVA in human urine.

The presence and identity of each analyte in human urine samples are verified by several criteria, which are: (a) urinary analytes comigrate with respective crystalline standards in HPLC analysis; (b) identical UV/visible scans are found for standards and urinary analytes; (c) spiking urine samples with DHPPAA, mHPAA, and HVA increases the expected peak.
heights in HPLC analysis; and (d) the mass spectra of urinary analytes match the mass spectra of respective standards. The detection of flavonoid metabolites in human urine samples is consistent with previous reports. Other investigators have found DHPAA and HVA in human urine samples (59, 60). Furthermore, similar concentrations of DHPAA and HVA are found in this and previous studies.

The HPLC-based method has several advantages over other analytical approaches. These advantages include that: (a) the method does not require derivatization of analytes; (b) the method is quantitative and sensitivity of the assay is high; (c) simultaneous extraction of multiple samples and automated HPLC analysis minimize technician time requirements for the assay; (d) only small amounts of urine (20 ml) are needed for the assay; (e) the assay is simple (it requires only two steps); (f) samples and standards are stable for at least 6 months with storage at −70°C; (g) unlike other assays, this assay measures mHPAA; and (h) the assay simultaneously measures DHPAA, mHPAA, and HVA.

Several aspects of the assay require attention for the maintenance of high-precision analysis. (a) Determination of internal standard recovery is essential. Low recoveries (<70%) generally indicate inadequate performance of the extraction procedure. Spurious data can result from analysis with low recoveries; i.e., p-coumaric acid may not be a perfect internal standard. Thus, we recommend reanalysis of samples with recoveries below 70%; this should be a low percentage of the samples. (b) If chromatograms are complex, the identity of each analyte should be verified by UV/visible scans. The scans can be done automatically with many scanning and photodiode detectors. (c) Peak broadening will occur without the use of a column-cleaning procedure. That is, adequate column cleaning is essential for optimal assay performance.

Application of the assay in studies of human populations requires consideration of concentration units. Significant day-to-day variations in urinary excretion volumes occur within and between subjects. Correction for urinary output is essential. Thus, urinary DHPAA, mHPAA, and HVA concentrations should be adjusted for total urinary output or creatinine concentrations. Another concern for application of the assay is the formation of DHPAA, HVA, and perhaps mHPAA from the metabolism of catecholamines and certain amino acids. For any individual, these metabolic pathways produce a relatively constant amount of DHPAA and HVA. Although dietary intake of quercetin can substantially increase DHPAA, mHPAA, and HVA over the amounts produced from catecholamines and amino acid metabolism, the best estimates of dietary determinants may involve experimental designs that incorporate measurements at two or more time points and evaluate changes in quercetin metabolite excretion. Between individuals, age, gender, drug use, body weight, and disease conditions may produce slight changes in HVA (62), and perhaps mHPAA and DHPAA excretion, and should be adjusted for in population studies.

Interpretation of results from human dietary studies should allow for the presence of multiple glycosides of quercetin. Most of the quercetin in foods exists in the form of glycosides; the most commonly occurring glycoside of quercetin is rutin. Rutin and other quercetin flavonols have similar metabolic profiles (63). Thus, multiple flavonols with an identical parent compound, quercetin, may yield several identical phenolic metabolites, such as HVA, DHPAA, and mHPAA.

Overall, the noted technical precautions for the assay are relatively minor, and the assay has numerous desirable characteristics. It has high sensitivity, precision, and analyte recoveries. The assay is quantitative and relatively simple. Automation of several steps in the assay allows for the simultaneous analysis of multiple samples. These characteristics make the routine analysis of DHPAA, mHPAA, and HVA in human studies feasible. This assay is the first analytical method that provides for the evaluation of DHPAA, mHPAA, and HVA as biomarkers in human studies. It is a practical method for screening human populations and the measurement of quercetin metabolites in human studies.

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**References**


The quantitation of metabolites of quercetin flavonols in human urine.

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