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

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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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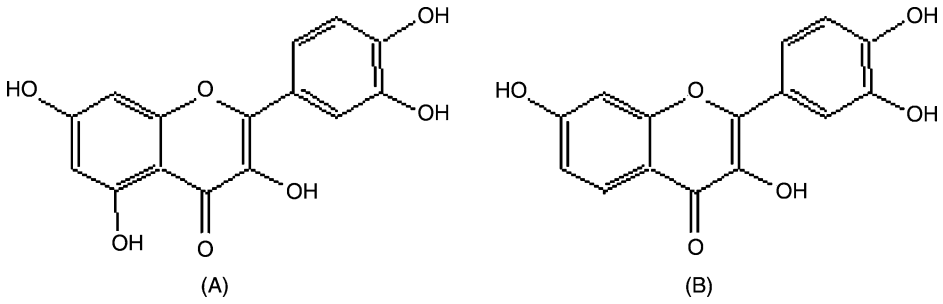

Fig. 1. Chemical structures of quercetin (A) and the internal standard fisetin (B).

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.

2.5. LC/MS/MS

A PE SCIEX API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystem, Foster City, CA) equipped with a TurboIonspray (TIS) interface, a series 2000 Perkin-Elmer pump, a series 2000 Perkin-Elmer autosampler (Shelton, CT) and Analyst 1.3 software was used for data acquisition and processing. Chromatographic separation of the analytes of interest was achieved on a C_{12} (particle size 5 um, 250 mm × 2.1 mm) column (Alltech) and the mobile phase consisted of acetonitrile/water with 0.2% formic acid (40/60, v/v). The flow rate was 200 μl/min; no post column splitting was performed and the injection volume was 10 μl. The mass spectroscopy was performed in a negative mode using multiple reaction monitoring. Optimal operating parameters of TIS were obtained, with respect to maximum signal intensity of molecular ions and fragment ions, by consecutive infusion of standard solutions of quercetin and fisetin (1 μg/ml) in mobile phases, using a Harvard syringe pump. The optimum conditions of the interface were as follows: ion spray voltage of −4500 V, pressure of collision gas (N_2) of 2.8 mTorr, flow rate of the nebulizer gas (air) and curtain gas (N_2) of 1.2 and 0.8 l/min, respectively. The interface temperature was set at 350 °C. The declustering, focusing and entrance potentials were −60 V, −300 V, and −10 V, respectively. The nebulizer current was 12 μA and fragmentation was induced with collision energy of −30 eV. Multiple reactions monitoring (MRM) of MS/MS was used for specific detection of the quercetin and fisetin (internal standard) by measuring the characteristic ion transitions of m/z 301.1 (parent ion) to *m*/*z* 151.1 (product ion) for quercetin and *m*/*z* 285.0 (parent ion) to m/z 135.1 (product ion) for the internal standard fisetin, respectively.

2.6. Calibration and validation

The standard curve was prepared over a concentration range of 1–800 ng/ml with nine different concentration levels. Standard curves were run on each analysis day and the coefficient of determination r^2 was used to judge linearity. Calibration was performed by an internal standard method. The integration was processed on Analyst software (Applied Biosystems, Foster City, CA) and calibration curves were obtained by plotting the extracting ion current (XIC) peak area ratios of analyte/internal standard versus concentrations using weighted linear regression.

Intra- and inter-day precision and accuracy and recovery from plasma and urine samples were assessed through triplicate analyses of the same samples containing known amounts of quercetin, with three samples per concentration level. Precision was evaluated as CV% of the mean of all the determinations at each concentration level. Accuracy was determined by comparing the calculated concentration to the known concentrations. Recovery was calculated by comparing the determined amounts for extracted blood or urine samples with the known amounts added. The limit of detection (LOD) was assessed as the quercetin concentration at a signal-to-noise ratio of 3:1. The lower limit of quantitation (LLOQ) was defined as the quercetin concentration yielding a mean assayed concentration within 20% of the known concentration and a precision with a CV% less than 20%.

2.7. Clinical study

To evaluate the usefulness of our assay for the evaluation of the disposition of quercetin in humans, we analyzed plasma and urine samples obtained from three subjects following the ingestion of Quercetin 500-Plus® capsules three times a day for 5 days. Informed consent was obtained and the study protocol was approved by the Investigational Review Boards at the University of Rochester and the University at Buffalo, State University of New York. Samples were analyzed before and after enzymatic hydrolysis to determine the concentrations of unchanged and conjugated quercetin metabolites present in plasma and urine samples.

3. Results/discussion

In this study, liquid chromatography-tandem mass spectrometry with a TSI interface in negative mode ionization with multiple reactions monitoring was used for the determination of quercetin in plasma and urine samples. A previous method by Wittig et al. [6] measured quercetin by LC/MS/MS using positive mode electrospray ionization and selective reaction monitoring, with a reported LOD of 2 ng on-column. This assay was used to successfully measure quercetin glucuronide conjugates following fried onion ingestion (equivalent to 100 mg quercetin), but was unable to detect any parent quercetin. Franke et al. [15] reported a liquid chromatographic-photodiode array mass spectrometric assay for the determination of quercetin after fried onion ingestion. They used negative mode after electrospray ionization with selective reaction monitoring and quantitated total quercetin (following enzymatic hydrolysis) in urine samples. Hong and Mitchell [8] recently determined a number of quercetin metabolites, but not unchanged quercetin, in human urine after onion ingestion using LC/ESI-MS/MS. Additionally, Mullen et al. [7] recently reported a LC assay for quercetin with detection by photodiode array and full scan tandem mass spectroscopy. Their analytical method utilized negative ion ESI with full scanning from *m*/*z* 100 to 1000. Using this assay, the authors were able to detect and identify a number of glucuronide and sulfate conjugates of quercetin in human plasma of subjects after the ingestion of fried red onions. One subject had trace levels of quercetin present in plasma, although the limit of detection of this assay for quercetin was not indicated.

Franke et al. [15] reported that negative mode ESI was superior to positive mode ESI for quercetin and Mullen et al. [7] also reported that negative ionization is about 10-times more sensitive than positive ionization for quercetin-3-glucuronide

when the metabolite is present in a urine sample. We also compared the TIS-MS behavior under both positive and negative modes for quercetin, finding that the negative ion mass spectrum was superior for the quantitation of quercetin under our conditions, providing better assay sensitivity.

3.1. LC/MS/MS conditions

For the LC separation, a C_{12} column was used in our assay because it gave less peak broadening and tailing, and shorter run times, compared with a C_{18} column. An acidic mobile phase (pH 2.4) using formic acid was found to provide optimal separation and quantification of quercetin (Fig. 2). Trifluoroacetic acid (TFA), acetic acid and formic acid were all evaluated for suitability under the mass spectrometric assay conditions. All are volatile, but the background noise using acetic acid was much higher than that using formic acid and TFA greatly suppressed the negative ion electrospray. Therefore, the addition of formic acid provided a much better signal-to-noise ratio and increased the sensitivity of the assay.

We found that acetone could effectively precipitate proteins without any co-precipitation of quercetin, which is consistent with previous reports [4,6], and this was used in our sample preparation. The internal standard used was the flavonoid, fisetin, since fisetin has the same mechanism of fragmentation and similar chromatographic characteristics as quercetin.

Usually atmospheric pressure chemical ionization (APCI) is used more extensively with small molecule compounds (MW < 1200), while ESI works better with higher molecular weight compounds. Theoretically APCI should be the best choice for quercetin, but experimentally, quercetin sensitivity with the ESI mode provided better sensitivity than that with the APCI mode. This is in agreement with the findings of Wittig et al. [6] and Franke et al. [15]. The possible reasons for this are that APCI can cause high chemical background noise for low mass compounds and thermal degradation can occur with labile compounds, such as quercetin, which would decrease the APCI sensitivity. Our assay used a TSI interface (the ionspray (IS) probe used in conjunction with a heated turboprobe) which aids in the desolvation of the sprayed

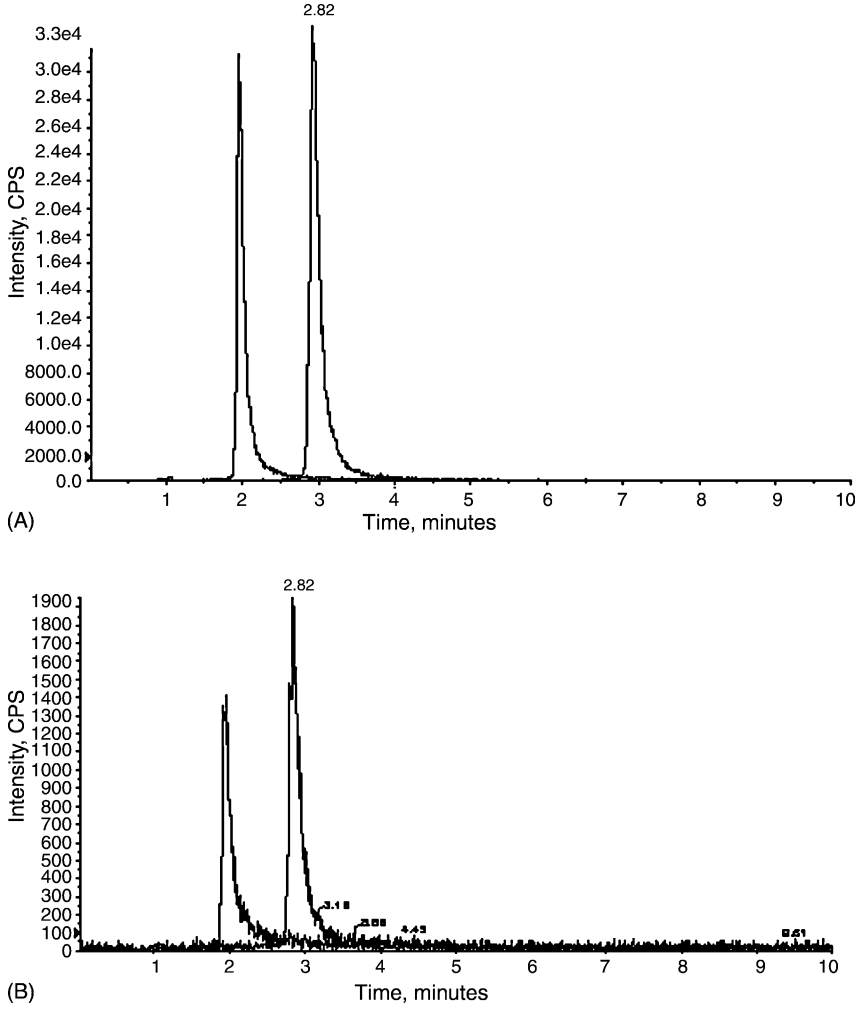

Fig. 2. Chromatograms of human plasma (A) and human urine (B) containing quercetin and the internal standard fisetin, obtained following the ingestion of quercetin 500 mg capsules. Quercetin has a retention time of 2.8 min and internal standard fisetin has a retention time of 2.0 min.

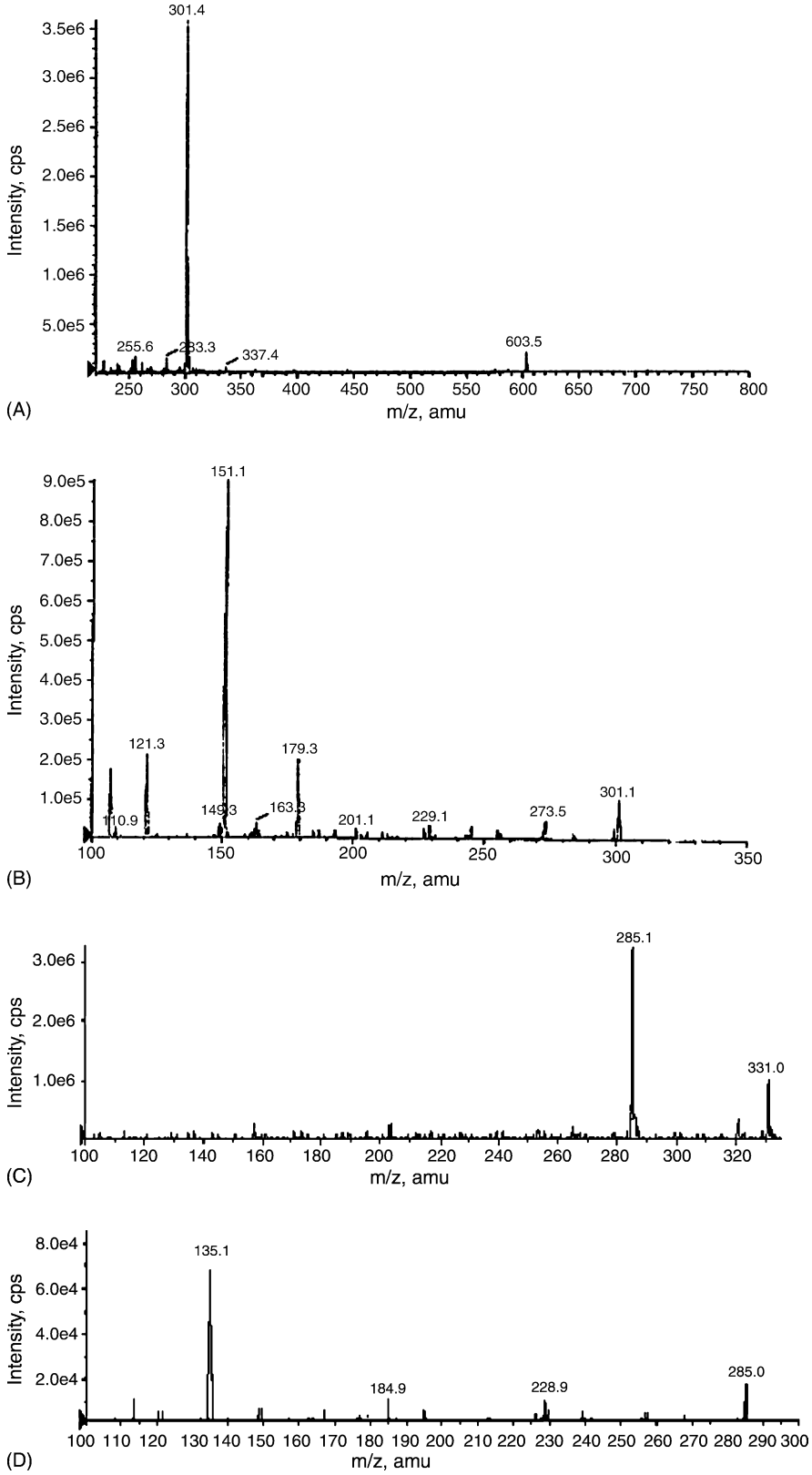

Fig. 3. Q1 full scan (A) and product ion scan (B) mass spectra of quercetin and Q1 full scan (C) and product ion scan (D) mass spectra of fisetin. The mass spectra were obtained by direct infusion of 1 μg/ml of quercetin and fisetin in acetonitrile/0.2% aqueous formic acid (2:3, v/v).

Table 1
Intra-day and inter-day accuracy and precision for quercetin in human plasma

	Nominal concentration (ng/ml)	Mean assayed concentration (ng/ml)	SD	Precision (CV%)	Accuracy (%)
Intra-day	1	1.12	0.11	9.64	112.4
	10	9.19	0.57	6.19	91.9
	100	96.5	7.04	7.30	96.5
	600	577	14.9	2.63	94.3
Inter-day	1	1.10	0.10	9.55	110
	10	8.82	0.30	3.40	88.2
	100	94.74	9.10	9.60	94.7
	600	565	26.3	4.65	94.2

Each individual value was the mean of triplicate determinations. The study was conducted over 3 days. Parameters were calculated as described in Section 2.

droplets. Unlike the thermospray interface, a TIS interface operates without the input of heat into the spray ionization step, so that labile and polar samples such as quercetin are ionized without thermal degradation.

The Q1 (first quadruple) full scan and product ion scan mass spectra of quercetin standard are presented in Fig. 3A and B, respectively. Molecular ion *m/z* 301.1 and 285 ($[M–H]^-$) were the most abundant ions for quercetin and the internal standard fisetin, respectively. The greatest product ion from the parent ion *m/z* 301.1 was *m/z* 151.1 for quercetin and from the parent ion *m/z* 285 was *m/z* 135.1 for fisetin (Fig. 3C and D). Therefore, the analyses were performed using MRM pairs of *m/z* 301.1 → 151.1 for the analyte and *m/z* 285 → 135.1 for internal standard fisetin. The retention times of quercetin and fisetin were 2.82 and 2.01 min (Fig. 3), respectively. The noise level was low with the intensity lower than 40 counts per second (cps). Due to the very high specificity of MS/MS, quercetin and fisetin can be unambiguously identified by their MRM pairs (301.1 → 151.1 and 258 → 135.1, respectively), even if they co-elute with other endogenous compounds.

3.2. Validation of the assay

Validation was performed with regard to LOD, LLOQ, linearity, intra- and inter-day precisions and accuracies in human plasma and urine. Based on five replicates assayed on three different occasions, the LOD was 100 pg/ml and the LLOQ was 500 pg/ml for plasma samples and the LOD was 500 pg/ml and the LLOQ was 1 ng/ml for urine samples. Compared with previous reports in the literature with LOQ values of 2.5 ng/ml [16] and 0.01–0.04 μg/ml [5], the present method provides better sensitivity. Our LOD is higher, though, than the LOD of 0.3 pg (60 pg/ml) and LLOQ of 1.5 pg (300 pg/ml) reported for a recent assay that utilized a semi-micro HPLC system with electrochemical detection [12].

Calibration curves of quercetin were linear over the concentration range of 1–800 ng/ml for plasma samples and 1–200 ng/ml and 50–2000 ng/ml for urine samples. Correlation coefficients of 0.99 or higher were obtained for the relationship between peak area ratios and the corresponding calibration concentration.

With regards to precision, CV% values for quercetin were less than 11% for inter-day and intra-day analysis (Tables 1 and 2). The accuracies for intra- and inter-day analysis were within ±15% of known concentrations (Tables 1 and 2).

3.3. Enzymatic hydrolysis

The optimal conditions for enzymatic hydrolysis of human plasma and urine samples were investigated by incubating the plasma and urine samples with different amounts

Table 2
Intra-day and inter-day accuracy and precision for quercetin in human urine

	Nominal concentration (ng/ml)	Mean assayed concentration (ng/ml)	SD	Precision (CV%)	Accuracy (%)
Intra-day	4	4.1	0.1	3.0	102.1
	100	97.1	1.6	1.7	97.1
	200	207.3	13.6	6.6	103.6
	1000	1013.8	23.0	2.3	101.4
	2000	1994.2	120.0	6.0	99.7
Inter-day	4	4.2	0.26	6.1	106.1
	100	92.4	3.9	4.2	92.4
	200	193.6	14.2	7.4	96.8
	1000	1009.4	103.5	10.3	100.9
	2000	2001.7	82.6	4.1	100.6

Each individual value was the mean of triplicate determinations. The study was conducted over 3 days. Parameters were calculated as described in Section 2.

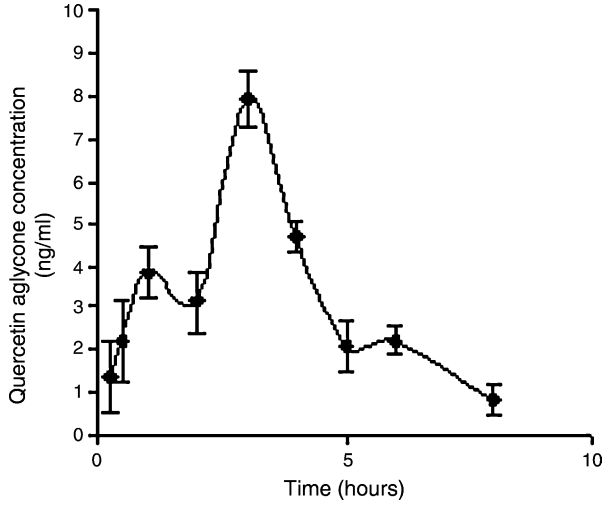

Fig. 4. Plasma concentration vs. time profile of quercetin aglycone in humans determined over an 8-h dosing interval at steady state, following the consumption of 500 mg quercetin three times daily. The data points are expressed as mean ± SE, $n = 3$.

of β-glucuronidase/sulfatase for different times. From these studies, the conditions were chosen in order to obtain the maximum amount of recovered quercetin (data not shown). The optimal conditions were incubation of human plasma samples with 5 μl glucuronidase (50 U/μl), 8 μl sulfatase (19.11 unit/ml) for 1 h and incubation of human urine with 10 μl glucuronidase (50 U/μl) and 20 μl sulfatase (19.11 unit/ml) for 1 h.

3.4. Clinical sample analysis

Plasma and urine samples were obtained over a dosing interval from three healthy human subjects receiving 500 mg quercetin three times a day. Unchanged quercetin could be determined in all plasma samples, with mean concentrations ranging from about 1 to 8 ng/ml (Fig. 4). In addition, total quercetin after β-glucuronidase and sulfatase hydrolysis was analyzed (Fig. 5). Following hydrolysis of the plasma samples, the mean total quercetin (unchanged plus conjugated) ranged from 120 to 350 ng/ml for plasma samples.

Urine samples contained unchanged and conjugated quercetin, with low amount of unchanged quercetin, 15.5, 27.9 and 74.9 μg excreted over 8 h for the three subjects.

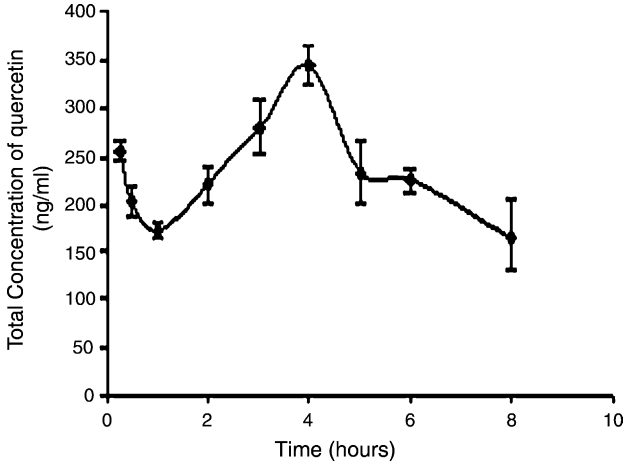

Fig. 5. Plasma concentration vs. time profile of total quercetin after β-glucuronidase and sulfatase hydrolysis of the plasma samples analyzed in Fig. 4.The data points are expressed as mean ± SE, $n = 3$.

4. Conclusions

The method described in this paper represents a highly sensitive and specific assay for the determination of both quercetin aglycone and total conjugated quercetin in human plasma and urine samples. To our knowledge, this is the first time quercetin aglycone was detected and quantitated in both human plasma and urine samples. The assay demonstrates a LOD of 100 pg/ml and intra- and inter-day coefficients of variation were 10% or less. The assay was successfully used to determine plasma and urine quercetin concentrations following the ingestion of 500 mg quercetin in humans, demonstrating peak plasma concentrations of 8 ng/ml.

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