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Journal of Chromatography B, 794 (2003) 49–56
JOURNAL OF CHROMATOGRAPHY B
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High-performance liquid chromatographic determination of quercetin in human plasma and urine utilizing solid-phase extraction and ultraviolet detection

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Received 2 August 2002; received in revised form 22 April 2003; accepted 7 May 2003

Abstract

An HPLC method for determining quercetin in human plasma and urine is presented for application to the pharmacokinetic study of rutin. Isocratic reversed-phase HPLC was employed for the quantitative analysis by using kaempferol as an internal standard. Solid-phase extraction was performed on an Oasis™ HLB cartridge (>95% recovery). The HPLC assay was carried out using a Luna ODS-2 column (150×2.1 mm I.D., 5 μm particle size). The mobile phase was acetonitrile–10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–glacial acetic acid, 29:70:1 (v/v, pH 3.9) and 26:73:1 (v/v, pH 3.9) for the determination of plasma and urinary quercetin, respectively. The flow-rate was 0.3 ml/min and the detection wavelength was set at 370 nm. Calibration of the overall analytical procedure gave a linear signal ($r > 0.999$) over a concentration range of 4–700 ng/ml of quercetin in plasma and 20–1000 ng/ml of quercetin in urine. The lower limit of quantification was ~7 ng/ml of quercetin in plasma and ~35 ng/ml in urine. The detection limit (defined at a signal-to-noise ratio of about 3) was ~0.35 ng/ml in plasma and urine. A preliminary experiment to investigate the plasma concentration and urinary excretion of quercetin after oral administration of 200 mg of rutin to a healthy volunteer demonstrated that the present method was suitable for determining quercetin in human plasma and urine.

Keywords: Quercetin

1. Introduction

Flavonoids occur naturally in the plant kingdom and generally are present as glycosides in the common human diet. Some epidemiological studies point to a potential effect of dietary flavonoids on the prevention of cardiovascular disease [1,2]. Quercetin (Fig. 1) is one of the most abundant flavonoids in vegetables and fruits [3]. This compound is mainly present as glycosides, such as quercetin-4′-glucoside in onion, quercetin-3-rutinoside (rutin) in tomato, and quercetin-3-galactoside in apple [4,5]. It has been considered that flavonoids supplied as glycosides are first hydrolyzed by the microflora before being absorbed [6]. Recently, Hollman et al. [7,8] speculated that some quercetin glycosides could be absorbed in humans and the bioavailability of various quercetin glycosides is affected by their sugar moiety. Mauri et al. [9] determined rutin in human plasma

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00398-2

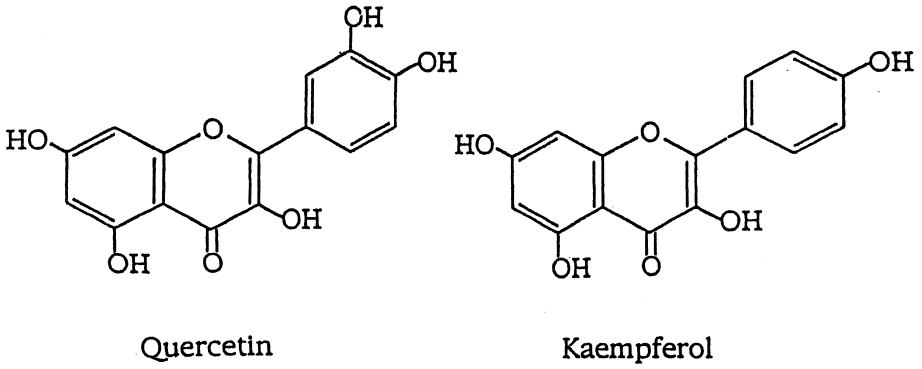

Fig. 1. Structures of quercetin and kaempferol (I.S.).

after subjects consumed tomato puree for 14 days. To characterize the precise pharmacokinetic properties of rutin, we have developed a method for the determination of rutin in human plasma. The method permitted the detection of trace amounts of rutin itself in human plasma after oral administration [10]. However, it is still not clear whether flavonoid glycosides are cleaved before or after absorption to the aglycones.

For a bioavailability study of flavonoid glycosides possessing quercetin as aglycone, the estimation of quercetin in biological fluids is essential. Several analytical methods suitable for the measurement of quercetin in biological fluids have been reported; HPLC–UV in plasma [11,12] and urine [13,14], HPLC with fluorometric detection in plasma and urine [15], HPLC–ED (electrochemical detection) in plasma [12,16], and a GC–MS method [17].

In this paper we developed a commonly available HPLC–UV method with solid-phase extraction, which showed sufficient specificity and simplicity for the measurement of quercetin in human plasma and urine.

2. Experimental

2.1. Chemicals and reagents

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) dihydrate and kaempferol (3,5,7,4′-tetrahydroxyflavone) were purchased from Extrasyntase (HPLC grade, purity >99%, Genay, France). Stock solutions of quercetin and kaempferol were prepared by dissolving these compounds in ethanol (purity 99.5%, Wako, Japan) followed by dilution with ethanol–water (50:50, v/v). Phosphoric acid was purchased from Aldrich (99.999%, Milwaukee, USA). β-Glucuronidase (from *Helix pomatia*, type H-1; β-glucuronidase activity 348 100 units/g, secondary sulfatase activity 11 603 units/g, Sigma Chemical Co., St. Louis, MO, USA). All other chemicals and solvents were used without further purification.

2.2. Sample preparation

To 1.0-ml aliquots of plasma were added different amounts of quercetin (3.6–714.8 ng) and a fixed amount (206.0 ng) of kaempferol as internal standard for the determination of quercetin in human plasma. For the determination of urinary quercetin, to 1.0-ml aliquots of urine different amounts of quercetin (17.9–1000.7 ng) and a fixed amount (206.0 ng) of kaempferol were added. The plasma or urine sample was diluted with 2 ml of 0.5 *M* phosphoric acid solution and then subjected to an Oasis™ HLB cartridge (30 mg packing, Waters, Milford, MA, USA). The cartridge was placed on vacuum manifold before loading the plasma or urine sample and conditioned by wetting with 1 ml of 0.5 *M* phosphoric acid solution. The sample was loaded at a flow-rate of 0.2 ml min^{-1}. The cartridge was first washed with 1 ml of 5% methanol in 0.5 *M* phosphoric acid solution, followed by 1 ml of 50% methanol in 0.5 *M* phosphoric acid solution. After purging with air, the

cartridge was eluted with 3 ml of methanol. The flow-rate of elution from the cartridge was 0.2 ml min^{-1}. After evaporating the eluate at 40 °C in vacuo, the residue was dissolved in 1 ml of methanol. The solution was transferred to a spitz tube with a ground-glass joint and evaporated to dryness at 40 °C in vacuo. The residue was dissolved in 20 μl of methanol with vortex-mixing for 30 s and then 100 μl of mobile phase with vortex-mixing for 30 s. A 30-μl portion of the solution was subjected to HPLC.

2.3. HPLC apparatus and conditions

HPLC analyses were performed on a Nanospace SI-2 (Shiseido, Tokyo, Japan) liquid chromatograph system equipped with a Model 3001 pump and a Model 3002 UV–Vis detector. The mobile phase was degassed with a Model 3009 degasser. Data processing was carried out with a Model 21 Sic chromatocorder (System Instrument, Tokyo, Japan). The HPLC system was consisted of a Luna C_{18} (2) (particle size 5 μm) column (150×2.00 mm, I.D.) (Phenomenex, Torrance, CA, USA) and a security guard cartridge (4×2.00 mm, I.D.) of the same material. The mobile phase was acetonitrile–10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–glacial acetic acid, the mixing volume ratio being 29:70:1 (pH 3.8) and 26:73:1 (pH 3.9) for the determination of plasma and urinary quercetin, respectively. The flow-rate was 0.3 ml min^{-1} and the detection wavelength was set at 370 nm. All HPLC analyses were performed at 20±1 °C.

2.4. Recovery

The absolute recovery was assessed at two concentration levels of 142.9 and 714.8 ng/ml for plasma quercetin or 71.5 and 714.8 ng/ml for urinary quercetin. The plasma and urine samples were applied to an Oasis™ HLB cartridge as described above. The peak-height ratios (quercetin to kaempferol as external standard) of the HPLC chromatograms were compared with those of reference solutions to calculate the absolute recoveries for quercetin in plasma or urine.

2.5. Calibration

Standard samples were prepared by adding known amounts of quercetin (3.6, 14.3, 71.5, 357.4, 500.4, 714.8 ng) to 1 ml blank plasma or (17.9, 71.5, 178.7, 357.4, 714.8, 1000.7 ng) to 1 ml blank urine. To each standard was then added 206.0 ng of kaempferol as the internal standard. The standard samples were prepared in duplicate. Standard curves were prepared following the extraction and HPLC analyses of the spiked plasma or urine samples. After determining the peak-height ratios (quercetin to kaempferol) of the HPLC chromatograms, the calibration graphs were obtained by least-squares linear fitting of the peak-height ratios versus the mixed mass ratios of quercetin to the internal standard.

2.6. Accuracy

Accuracy was determined by assaying in duplicate six preparations of 1.0-ml aliquots of human plasma containing three different amounts of quercetin (7.1, 428.9, 643.3 ng) for plasma quercetin or for urinary quercetin (35.8, 500.4, 929.5 ng) and a fixed amount (206.0 ng) of kaempferol as the internal standard. After preparing the samples for HPLC as described above, the peak-height ratios were determined.

2.7. Stability in plasma or urine

Stability of quercetin in plasma or urine was examined at 37 °C and −20 °C. To the plasma or urine sample was added quercetin (500.4 ng/ml) and the sample was incubated at 37 °C for 1, 2, 4, 6 and 24 h. Storage stability at −20 °C was determined after storing the plasma or urinary sample containing quercetin of 500.4 ng/ml for 3 days. For the stability of quercetin in biological fluids under the antioxidant reagent, 100 μl of 10% ascorbic acid solution were added to 1 ml of plasma (containing 50.0 ng, 500.4 ng/ml quercetin) or urine (containing 71.5 ng, 357.5 ng/ml quercetin) sample and the biological sample was stored for a period of one to 4 weeks at −20 °C.

2.8. Enzymatic hydrolysis

For the determination of quercetin glucuronides and sulfates, 1.0-ml aliquots of plasma or urine

containing ~1% of ascorbic acid as an antioxidant reagent were hydrolyzed by β-glucuronidase according to the method of Axelson et al. [18]. An enzyme solution (5 ml) from *H. pomatia* containing 10 000 activity units of β-glucuronidase and 333 activity units of sulfatase in 0.2 *M* acetate buffer solution (pH 4.5) and a fixed amount (206.0 ng) of kaempferol as an internal standard was added to 1.0 ml plasma or urine sample. The reaction mixture was incubated at 37 °C for 24 h. After 20 ml of ethanol were added to the mixture, the solution was vortexed for 1 min and centrifuged at 3000 rpm for 5 min. The resulting supernatant was evaporated. The residue was then dissolved in 3 ml of 0.5 *M* phosphoric acid solution and extracted with an Oasis™ HLB cartridge as described above.

2.9. Application of the assay and sample collection

A single 200-mg amount of rutin was orally administered to a 48-year-old healthy male volunteer. Plasma samples (3 ml each) were collected just before and at 2, 4, 5, 6, 8 and 24 h after administration. Urine samples were collected just before and at 1, 2, 7.5, 8, 11.3, 17.5 and 22.5 h after administration.

3. Results and discussion

In a biological matrix, a clean-up step prior to HPLC–UV analysis is generally required for successful analysis. Previously, a Sep-Pak C_{18} cartridge, an anion-exchanger Sep-Pak Accell™QMA cartridge, and an Oasis™ MAX cartridge were used for the clean-up of several flavonoids from a biological matrix [19–22]. For quercetin in human plasma and urine, however, the use of these cartridges was found to be unsatisfactory. The use of an Oasis™ HLB cartridge effectively eliminated the interfering material in plasma and urine with efficient extraction of quercetin. The cartridge was washed twice, first with 5% methanol and second with 50% methanol in 0.5 *M* phosphoric acid solution. Although only the first wash step efficiently cleaned up the sample from interesting material in the HPLC chromatogram, the second step was necessary to eliminate compounds accumulating on the HPLC column. Methanol was used to elute quercetin from the Oasis™ HLB cartridge. For calculating the absolute recoveries of quercetin from human plasma or urine, the peak height ratios [quercetin to kaempferol (Fig. 1) as external standard] of the HPLC chromatograms were compared with those of reference solutions. The recoveries were 96.5±6.9% (142.9 ng/ml, $n=3$) and 95.3±4.2% (714.8 ng/ml, $n=3$) for plasma quercetin. For urinary quercetin, the recoveries were 96.9±3.9% (71.5 ng/ml, $n=3$) and 98.4±4.4% (714.8 ng/ml, $n=3$).

The HPLC behavior of quercetin extracted from human plasma or urine was isocratically examined by using a Luna C_{18} (2) column. The use of a solvent system consisting of acetonitrile–10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–glacial acetic acid (29:70:1, v/v, pH 3.9) was found to provide good chromatographic profiles for quercetin and kaempferol (I.S.) for the determination of plasma quercetin. For urinary quercetin, the mobile phase consisted of acetonitrile –10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–glacial acetic acid (26:73:1, pH 3.9). Figs. 2A and 3A show typical HPLC chromatograms of human plasma (Fig. 2A) and urine (Fig. 3A) without spiking quercetin and the internal standard. Fig. 2B illustrates a chromatogram of an extract of human plasma spiked with quercetin (7.1 ng, at the lower

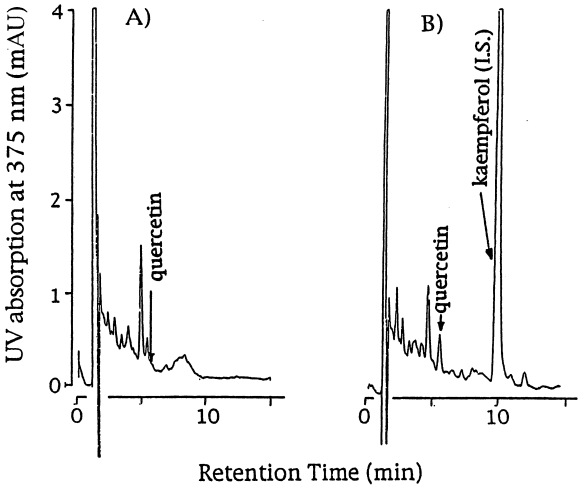

Fig. 2. HPLC chromatograms of extracts of (A) blank plasma and (B) plasma spiked with quercetin (7.1 ng) and kaempferol (206.0 ng, I.S.).

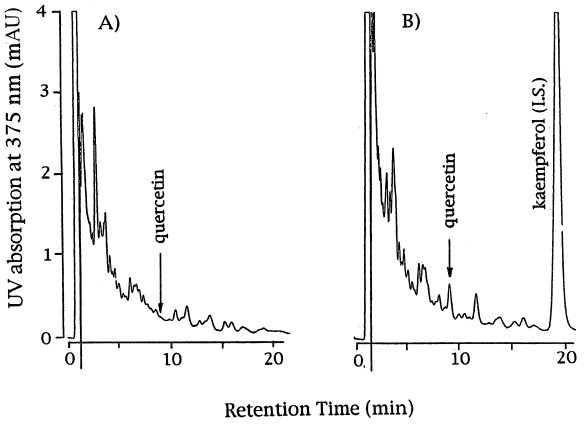

Fig. 3. HPLC chromatograms of extracts of (A) blank urine and (B) urine spiked with quercetin (17.9 ng) and kaempferol (206.0 ng, I.S.).

limit of quantification, LOQ) and kaempferol (206.0 ng, I.S.). Fig. 3B illustrates a chromatogram of a urine extract spiked with quercetin (17.9 ng, at lower end point of calibration graph) and kaempferol (206.0 ng, I.S.). A comparison of the chromatograms shown in Figs. 2 and 3 demonstrates that there is no significant interference from endogenous components in the analysis of plasma and urinary quercetin.

Calibration graphs were prepared by using 1.0-ml aliquots of the blank pooled plasma or urine spiked with different amounts of quercetin ranging from 3.6 to 714.8 ng for plasma quercetin or from 17.9 to 1000.7 ng for urinary quercetin and 206.0 ng of kaempferol as internal standard. The peak-height ratios were plotted against the mixed mass ratios of quercetin to the internal standard. A good correlation was found between the observed peak-height ratios (y) and mixed mass ratios (x). A least-squares regression analysis gave a typical regression line of $y = 0.0066x + 0.00005$ ($r > 0.999$) for plasma quercetin and $y = 0.0067x - 0.0977$ ($r > 0.999$) for urinary quercetin.

The accuracy of measurements was determined in duplicate by adding 7.1, 428.9 and 643.3 ng of quercetin to 1.0-ml aliquots of plasma or 35.8, 500.5 and 1000.7 ng of quercetin to 1.0-ml aliquots of urine with a fixed amount of kaempferol (206.0 ng). Table 1 shows that the amounts of quercetin added were in good agreement with the amounts of quercetin measured, the relative errors being less than 4% (428.9 ng/ml plasma in the midrange, 643.3 ng/ml plasma at the high end of the range, 35.8 ng/ml urine at the near the LOQ, 500.5 ng/ml urine in the midrange). Larger relative errors were observed for 7.1 ng/ml plasma at the near the LOQ (−8.5%) and 929.5 ng/ml urine at the end of the range (+8.3%). The inter-assay relative standard deviations (RSDs) ($n = 6$) were less than ±4% except low-quercetin in plasma (9.4% for 7.1 ng/ml). The limit of quantification was defined as the lowest concentration of quercetin that could be measured with an acceptable accuracy and precision (RSD<10%). The limit of quantification was 7.1 ng/ml for quercetin in plasma. The limit of quantification was 35.8 ng/ml for quercetin in urine, because the accuracy for 21.5 ng/ml urine was only 37.1% ($n = 6$). The LOD of the present HPLC assay (defined as a signal-to-noise ratio of about 3) was ~0.35 ng/ml plasma or urine, which the ratio was about ~8 on HPLC chromatogram of the water extract containing quercetin Fig. 4.

The stability of quercetin in a blood-collection

Table 1
Accuracy of HPLC determination of quercetin in human plasma and urine

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D (%)
	Individual values						Mean±SD		
Plasma									
7.1	6.2	5.6	6.6	7.4	6.5	6.9	6.5±0.6	−8.5	9.4
428.9	456.4	449.2	441.6	439.9	438.9	446.9	445.5±6.7	+3.9	1.5
643.3	601.2	610.8	631.0	647.0	671.0	605.5	627.8±27.3	−2.4	4.4
Urine									
35.8	35.9	36.7	37.4	35.7	38.4	37.8	37.0±1.1	3.3	2.1
500.5	486.7	511.8	528.5	511.0	486.1	497.7	503.6±15.0	−0.6	3.3
929.5	985.4	1015.4	1007.9	1026.1	1030.7	975.6	1006.8±22.1	8.3	2.2

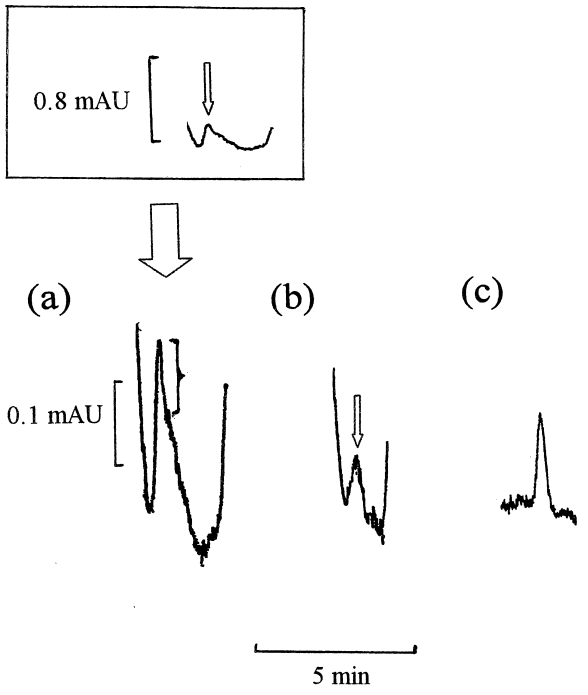

Fig. 4. Detection limits for quercetin (0.35 ng/ml) in plasma (a), urine (b) and water (c). Mobile phase: acetonitrile–10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–glacial acetic acid; (a) 29:70:1 (v/v) (quercetin RT, 5.7 min); (b) and (c) 26:73:1 (v/v) (quercetin RT, 9.3 min). The peak for quercetin (0.35 ng/ml) in plasma (a) corresponds to the brace part on the top of an ambiguous interfering background.

tube containing EDTA or plasma was previously studied in detail by Erlund et al. [16]. These authors found that no loss of quercetin in plasma was detected in samples frozen once at −70 °C, though quercetin was unstable under various conditions. In our experiments when the plasma or urine samples were stored at −20 °C and incubated at 37 °C, the quercetin was also found to be unstable. A large portion of quercetin in the samples was lost (~95% of the initial amount in 24 h at 37 °C, ~35% in 3 days at −20 °C). When 100 μl of 10% ascorbic acid solution as an antioxidant reagent was added to 1.0 ml of plasma or urine and stored, quercetin in these biological fluids was found to be stable for at least 1 month at −20 °C and for 24 h at 37 °C.

As an example of the application of the present method, the plasma concentrations and the urinary excretion of quercetin and quercetin glucuronides and sulfates were investigated in a healthy volunteer who received orally 200 mg of rutin. A time course of plasma concentrations of quercetin measured by the present HPLC method after hydrolysis of its glucuronides and sulfates is shown in Fig. 5A. In the blood sample collected just before the intake of 200 mg rutin, 17.4 ng/ml of quercetin was detected. There was a relatively slow rise in the plasma concentration of quercetin to 34.7 ng/ml at the first blood sampling time of 2 h after the oral administration (HPLC chromatogram is shown in Fig. 6) giving a peak level of 142.7 ng/ml at 5 h. From the decline phase of semilogarithmic plots of the plasma concentration of quercetin versus time, the elimination half-life ($t_{1/2}$) of quercetin was calculated to be approximately 20 h. In a previous rutin supplementation study reported by Hollman et al. [23], the plasma concentrations of quercetin and its conjugates were measured. It was observed that the peak plasma levels of quercetin in human volunteers were at 9 h (with a lag-time of 4 h) after the administration, indicating absorption from the colon. In our data, the absorption lag-time was not observed. It has also been suggested that quercetin circulates in plasma only in its conjugated form [23–25]. In our study, however, free quercetin (13.8 ng/ml) was detected only in the plasma sample taken at 8 h after the rutin administration. The 24-h cumulative urinary excretion of quercetin, quercetin glucuronides and sulfates shown in Fig. 5B demonstrated that the cumulative amounts gradually increased during the 24-h urine collection period after oral administration of 200 mg of rutin to the subject. The percentage of quercetin and its conjugates excreted in urine after ingestion of rutin was 0.30% (free quercetin: 0.31 μmol, conjugates: 0.69 μmol) of the administered dose (200 mg rutin: 328 μmol) within 24 h.

This paper describes a commonly available HPLC–UV method with solid-phase extraction for the measurement of quercetin in human plasma and urine. HPLC–UV methods developed by other workers did not provide good sensitivity for determining low levels of plasma quercetin (LOD, 80–100 ng/ml plasma) [11,12]. Bongaetz and Hesse [13] used boronic acid affinity chromatography to determine urinary quercetin in a concentration range of 0.10–30.0 μg/ml urine. Better sensitivity was obtainable

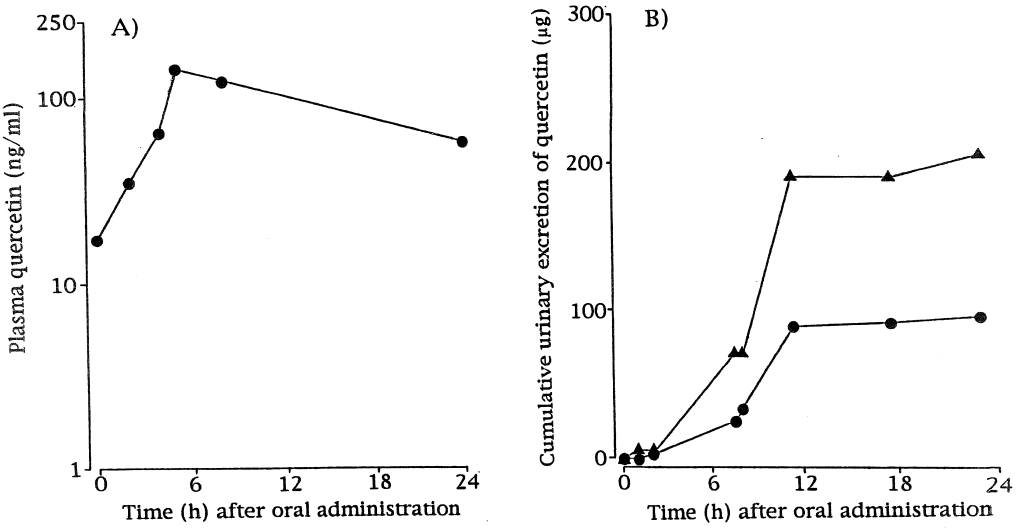

Fig. 5. Plasma concentration of quercetin (ng/ml) after hydrolysis of its glucuronides and sulfates (A) and cumulative urinary excretion of quercetin (B, –▲–, glucuronides and sulfates; –•–) after intake of rutin (200 mg) by a healthy volunteer.

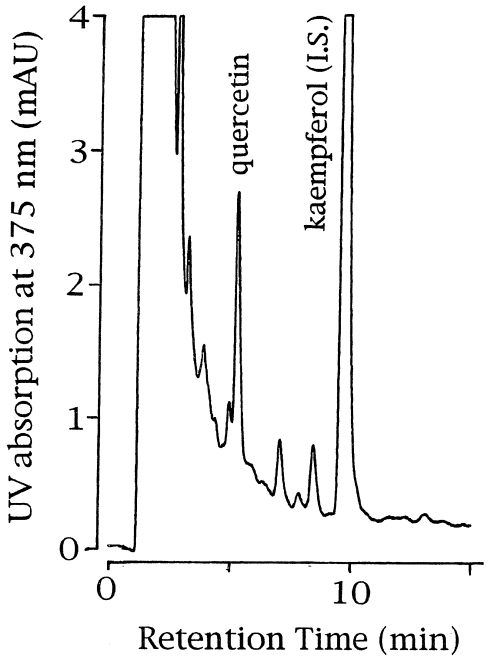

Fig. 6. HPLC chromatogram of the enzymatic hydrolysis plasma extract at 2 h after the oral administration.

by HPLC methods with fluorometric detection using postcolumn derivatization [15] or with electrochemical detection (ED) [12], the LOD being 2 ng/ml plasma or urine. Using HPLC–ED, Erlund et al. [16] obtained a low quantification limit of 0.63 ng/ml plasma. Nielsen et al. [14] found difficulties in quantifying quercetin in human urine by using HPLC–ED, due to interfering peaks presented in the ED chromatogram, and developed a column-switching HPLC method for detecting low levels of quercetin by using UV absorbance detection. The sensitivity of this HPLC–UV method (LOD, 5 ng/ml urine) was similar to that of the above mentioned HPLC with fluorometric detection [15] and HPLC–ED [12].

The detection limit of the present method was ~0.35 ng/ml plasma or urine and the LOQ was ~7 ng/ml of quercetin in plasma and ~35 ng/ml in urine. The method also provided sufficient specificity and the analytical time required for quercetin in plasma and urine was relatively short (about 20 min) compared with other HPLC method [12,15]. Furthermore, the present method is advantageous in that the same analytical procedures can be used for measuring quercetin in the plasma and urine samples. The method is suitable for pharmacokinetics studies with sufficient specificity, simplicity and sensitivity.

Acknowledgements

This work was supported, in part, by a Project Research Grant of Kyorin University.

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