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Journal of Chromatography B, 759 (2001) 161–168

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of rutin in human plasma by high-performance liquid chromatography utilizing solid-phase extraction and ultraviolet detection

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Received 3 October 2000; received in revised form 29 January 2001; accepted 18 April 2001

Abstract

An HPLC method for determining a flavonol glycoside, rutin, in human plasma is presented for application to the pharmacokinetic study. Isocratic reversed-phase HPLC was employed for the quantitative analysis by using kaempferol-3-rutinoside as an internal standard. Solid-phase extraction was performed on an Oasis MAX cartridge possessing reversed-phase and anion-exchange functions (recovery, approximately 80%). 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 (16.5:82.5:1, v/v, pH 3.8). The flow-rate was 0.3 ml/min. The detection wavelength was set at 370 nm. Calibration of the overall analytical procedure gave a linear signal ($r>0.9999$) over a concentration range of 3–1000 ng/ml of rutin in plasma. The lower limit of quantification was ca. 5 ng/ml of rutin in plasma. The detection limit (defined as signal-to-noise ratio of about 3) was approximately 0.75 ng/ml. A preliminary experiment to investigate the plasma concentration of rutin after oral administration of 500 mg of rutin to a healthy volunteer demonstrated that the present method was suitable for determining rutin in human plasma.

Keywords: Rutin

1. Introduction

Flavonoids occur naturally in the plant kingdom and are contained in the common human diet. These flavonoids have been shown to possess many biological and pharmacological activities, such as antioxidant activity [1–4], tumor growth-inhibitory activity in various cancer cell lines [5–7] in vitro, and reducing effect against the risk of the breast cancer [8].

Flavonoids usually occur as glycosides in dietary plants. There has been little information concerning the extent to which the flavonoids are absorbed in humans, their metabolism, pharmacokinetics and bioavailability. It has been considered that flavonoids supplied as glycosides are first hydrolyzed by the microflora before being absorbed [9]. Recent studies demonstrated that flavonoid glycosides were normal constituents in human plasma by determining either

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00224-9

intact flavonoid glycosides directly [10] or their aglycone moieties obtained by acid hydrolysis of biological samples after dietary intake [11]. However, it is still not clear whether the glycosides are cleaved before or after absorption to generate the aglycones.

A flavonol glycoside, rutin (quercetin-3-rutinoside, Fig. 1), which occurs in common vegetables [12,13], has an antihemorrhagic activity in blood vessels [14,15]. This bioflavonoid was also suggested to act as a potent radical scavenger [16].

To characterize the precise pharmacokinetic properties of rutin, it is important to develop a highly sensitive and simple analytical method for quantitating rutin in its intact flavonol glycoside form. Reversed-phase high-performance liquid chromatography (HPLC) has been widely utilized for the purification and separation of naturally occurring flavonoids in crude plant materials or food products [17–21] and biological fluids [22–24]. Several recent reports describe the analysis of rutin in human plasma by HPLC with photodiode array detection (DAD) [10] and by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) [25]. These methods, however, do not provide satisfactory sensitivity for pharmacokinetic studies.

In this paper we report the development of a HPLC–UV method which shows sufficient specificity, sensitivity and simplicity for the measurement of rutin in human plasma.

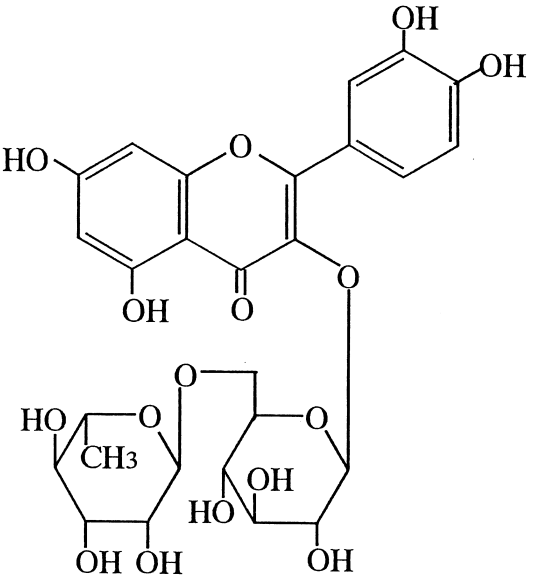

Fig. 1. Structure of rutin.

2. Experimental

2.1. Chemicals and reagents

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside, quercetin-3-rutinoside) and kaempferol-3-rutinoside (3,4′,5,7-tetrahydroxyflavone-3-rutinoside) were purchased from Extrasyntase (HPLC grade, Genay, France). Stock solutions of rutin and kaempherol-3-rutinoside were prepared by dissolving these compounds in methanol, followed by dilution with water of equal volume (methanol–water, 50:50, v/v). All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Sample preparation

To 1.0-ml aliquots of plasma were added different amounts of rutin (3.14–1047.29 ng) and a fixed amount (197.50 ng) of kaempherol-3-rutinoside as internal standard for the determination of rutin in human plasma. The plasma sample was diluted with 2 ml of water and applied to a lipophilic anion exchanger Oasis MAX cartridge (30 mg packing, Waters, Milford, MA, USA) without preconditioning. The cartridge was first washed with 5 ml of water, followed by 1 ml of methanol and 1 ml of acetonitrile. After purging with air, the cartridge was eluted with 1 ml of a mixed solution of 100 m*M* phosphoric acid–acetonitrile (50:50, v/v). The flow-rate of elution from the cartridge was ca. 2 ml/min. After evaporating the eluate at 40°C in vacuo, the residue was dissolved in 1 ml of ethanol and the solution was filtered through a HLC-Disc filter (Kanto Chemicals, Tokyo, Japan). The filtrate 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 60 μl of the 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 Jasco (Tokyo, Japan) liquid chromatograph equipped with a Model PU-1580 pump and a Model UV-1570 detector. The mobile phase was degassed with a Model DG-980-51 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 (150×2.1 mm I.D.) (Phenomenex, Torrance, CA, USA) and a security guard cartridge (4×2 mm I.D.) of the same material. The mobile phase was acetonitrile–10 m*M* ammonium acetate solution containing 300 m*M* EDTA–glacial acetic acid (16.5:82.5:1, v/v, pH 3.8). The flow-rate was 0.3 ml/min. The detection wavelength was set at 370 nm.

2.4. LC–ESI-MS apparatus and conditions

The HPLC apparatus and chromatographic conditions were appropriately designed for LC–ESI-MS and MS–MS analyses. The HPLC apparatus used was a Waters liquid chromatograph controlled by a Model 600S controller equipped with a Model 616 pump, a Model 486-MS tunable absorbance detector, a Model 616 pump, a Model 486-MS tunable absorbance detector, and a Model 21 Sic chromatocorder for data processing. The mobile phase was degassed with a Waters In-Line degasser. A Luna C_{18}(2) (particle size 3 μm) column (100× 1.00 mm I.D.) (Phenomenex) was employed. The mobile phase was acetonitrile–10 m*M* ammonium acetate (17:83, v/v), and the flow-rate was 0.05 ml/min. The detection wavelength was set at 370 nm. The mass spectral data were collected with a quadrupole ion trap mass spectrometer (Finnigan LCQ instrument, San Jose, CA, USA) equipped with a heated capillary electrospray interface. The ion polarity mode was set to the positive MS and MS–MS mode. The sprayer needle voltage was 4.5 kV with a nebulizer gas flow set at 75% of the maximum. The temperature of the heated capillary was 195°C. Capillary and tube lens voltages were 10 V and 0 V, respectively. The total microscan was 2 microscans. The collision gas pressure was 10^{-3} Torr (1 Torr=133.322 Pa). The relative collision energies for the MS–MS mode analyses were optimized at 35% of the maximum setting (5 V). The MS–MS spectra were obtained with a precursor ion isolation width of 2 *m/z*.

2.5. Recovery

The absolute recovery for rutin was assessed at two concentration levels of 157.09 and 1047.29 ng/ml in plasma. The plasma samples were applied to an Oasis MAX cartridge as described above. The peak-height ratios (rutin to kaemperol-3-rutinoside as external standard) of the HPLC chromatograms were compared with those of reference solutions to calculate the absolute recoveries for rutin.

2.6. Calibration

Standard samples were prepared by adding known amounts of rutin (3.14, 10.48, 41.90, 628.37, 1047.30 ng) to 1-ml blank plasma. To each standard was then added 197.50 ng of kaempherol-3-rutoinoside 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 samples. After determining the peak-height ratios (rutin to kaempherol-3-rutinoside) of the HPLC chromatograms, the calibration graphs were obtained by a least-squares linear fitting of the peak-height ratios versus the mixed mass ratios of rutin to the internal standard.

2.7. Accuracy

Accuracy was determined for rutin by assaying six preparations of 1.0-ml aliquots of human plasma containing three different amounts of rutin (5.23, 523.60 and 942.56 ng) and a fixed amount (197.50 ng) of kaempherol-3-rutinoside as the internal standard in duplicate. After preparing the samples for HPLC as described above, the peak-height ratios were determined.

2.8. Stability in plasma

Stability of rutin in plasma was examined at 37°C and −20°C. To the plasma samples was added rutin

(41.90 ng/ml) and the sample was incubated at 37°C for 1, 2, 3, 4, 5 and 24 h. Storage stability at −20°C was determined after storing the samples (containing 41.90 ng/ml and 628.37 ng/ml, in duplicate) over a period of 30 days.

2.9. Application of the assay and sample collection

After a single oral administration of 500 mg of rutin to a 46-year-old male healthy volunteer, plasma samples (4 ml each) were collected just before administration and at 2, 4, 5, 6, 8, 10, and 24 h.

3. Results and discussion

In an attempt to conduct pharmacokinetic studies of three flavonoid glycosides, naringin, rhoifolin and daidzin, we have already described methods for the determination of these glycosides in human plasma [26,27] and urine [28,29] by reversed-phase HPLC using an ODS column. The clean-up of required components in biological matrix prior to HPLC–UV analysis is a prerequisite for the successful analysis, especially in the case of highly polar components in biological fluids. In our previous work, a Sep-Pak C_{18} cartridge or a anion exchanger, Sep-Pak Accell QMA cartridge, was used for the clean-up of these flavonoids from the biological matrix. For rutin in human plasma, however, the use of these cartridges and an Oasis HLB cartridge was found to be unsatisfactory. The use of an Oasis MAX cartridge with reversed-phase and anion-exchange functions effectively eliminated the interfering material in plasma with efficient extraction of rutin.

A mixed solution of 100 m*M* phosphoric acid–acetonitrile (50:50, v/v) was used to elute rutin from the Oasis MAX cartridge. For calculating the absolute recoveries of rutin from human plasma, the peak height ratios (rutin to kaempferol-3-rutinoside as external standard) of the HPLC chromatograms were compared with those of reference solutions. The recoveries were 79.7±1.3% (157.09 ng, $n=3$) and 80.3±4.2% (1047.29 ng, $n=3$) for rutin.

The HPLC behavior of rutin extracted isocratically from human plasma was examined by using a Luna $C_{18}(2)$ column. The use of a solvent system consisted of acetonitrile–10 m*M* ammonium acetate solution containing 0.3 m*M* EDTA–acetic acid (16.5:82.5:1, v/v, pH 3.8) was found to provide good chromatographic profiles of rutin and kaempferol-3-rutinoside (I.S.). For the future analysis of rutin by LC–ESI-MS, the mobile phase containing ammonium acetate was used. The addition of EDTA to the mobile phase prevented the peak tailing of the polyphenols possessing catechol structure [30]. Fig. 2A shows a typical HPLC chromatogram of human plasma without spiking rutin and the internal standard. Fig. 2B illustrates the chromatogram of an extract of human plasma spiked with rutin (5.23 ng, at near the lower limit of quantification, LOQ) and kaempferol-3-rutinoside (197.50 ng, I.S.). Comparison of the chromatograms shown in Fig. 2A and Fig. 2B demonstrates that there is no significant interference from endogenous components for the analysis of rutin.

Calibration graphs were prepared by using 1.0-ml aliquots of the blank pooled plasma spiked with different amounts of rutin ranging from 3 to 1000 ng and 197.50 ng of kaempferol-3-rutinoside as the internal standard. The peak-height ratios were plotted against the mixed mass ratios of rutin to the internal standard. A good correlation was found between the observed peak-height ratios (y) and the mixed mass ratios (x). A least-squares regression analysis gave a typical regression line of $y=0.0098x-0.0040$ ($r>0.999$) for rutin.

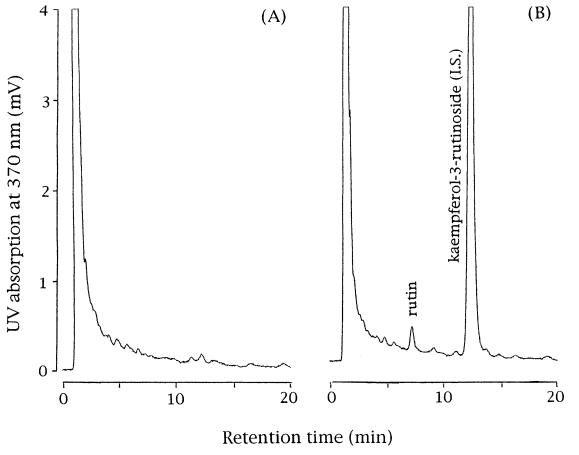

Fig. 2. HPLC chromatograms of extracts of (A) blank plasma and (B) plasma spiked with rutin (5.23 ng) and kaempferol-3-rutinoside (197.50 ng, I.S.).

Table 1
Accuracy of HPLC determination of rutin in human plasma

Added (ng/ml)	Found (ng/ml)							Relative error (%)	RSD (%)
	Individual values						Mean±SD		
5.23	5.91	5.31	6.25	5.42	6.40	5.16	5.88±0.42	+6.69	7.45
10.48	10.35	40.46	11.02	10.55	10.52	10.37	10.58±0.42	+0.95	2.31
523.60	530.18	517.02	526.07	509.74	507.55	512.71	520.37±9.14	−0.60	1.76
942.56	916.83	903.37	942.72	931.70	926.83	943.86	927.72±15.63	−1.57	1.69

The accuracy of measurements was determined by adding 5.23, 523.60 and 942.56 ng of rutin to 1.0-ml aliquots of plasma in duplicate with a fixed amount of kaemoferol-3-rutinoside (197.50 ng). Table 1 shows that the amounts of rutin added were in good agreement with the amounts of rutin measured, the relative errors being less than ±2% for 523.60 and 942.56 ng/ml and 4% for 5.23 ng/ml. The inter-assay relative standard deviations (RSDs) ($n=6$) were less than 2% for 523.60 and 942.56 ng/ml and 8% for 5.23 ng/ml. The intra-assay RSDs were less than 2% (523.60 ng/ml, $n=6$ and 942.56 ng/ml, $n=5$), and 9% (5.23 ng/ml, $n=6$). The precision (RSDs) and the accuracy (R.E.) at the three concentrations (5.23 ng/ml at the near the LOQ, 523.60 ng/ml in the midrange, and 942.56 ng/ml at the high end of the range) were acceptable in view of the international recommendations [31]. The sensitivity of the present HPLC assay (defined as a signal-to-noise ratio of about 3) was 0.75 ng/ml plasma for rutin. The sensitivity of this method for determining rutin in human plasma is higher than that of the reported HPLC–DAD method [10] (244–366 ng/ml) or LC–ESI-MS [25] method (300 ng/ml).

Rutin added to plasma samples was found to be stable for at least 24 h at 37°C. When stored at −20°C, it was stable for at least 1 month.

As an example of the application of this method, the plasma rutin was analyzed in the plasma from a healthy volunteer who orally received 500 mg of rutin. Fig. 3 shows HPLC chromatograms of plasma extracts at different time points after the oral administration. It was observed that the rutin peaks on the chromatograms slowly increased up to 5 h and decreased thereafter.

The positive ion ESI mass spectrum of authentic rutin (40 ng) (Fig. 4A, left) showed an abundant ion peak at m/z 611, which was the proton adduct of

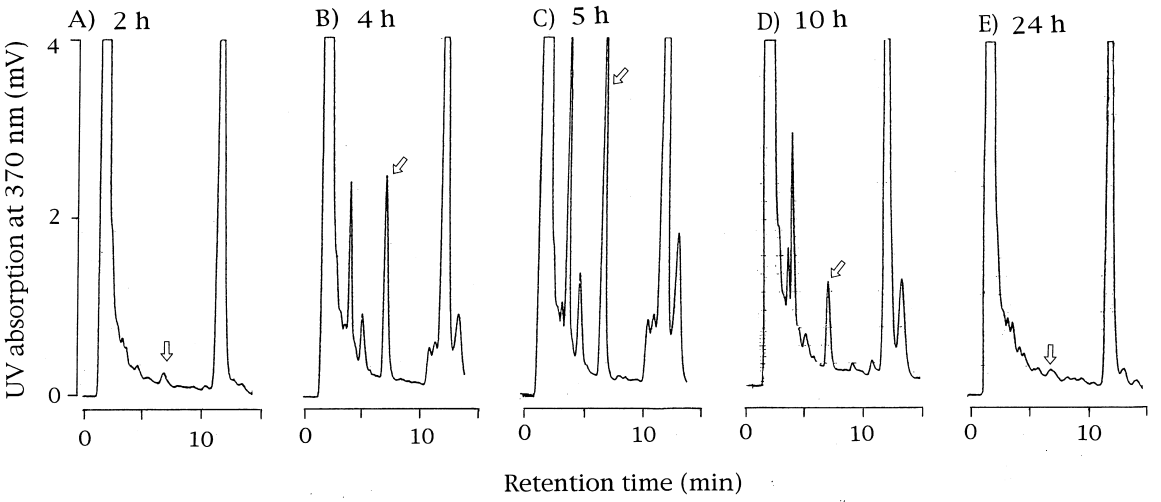

Fig. 3. HPLC chromatograms of plasma extracts at different time points (A: 2 h, B: 4 h, C: 5 h, D: 8 h, E: 24 h) after the oral administration.

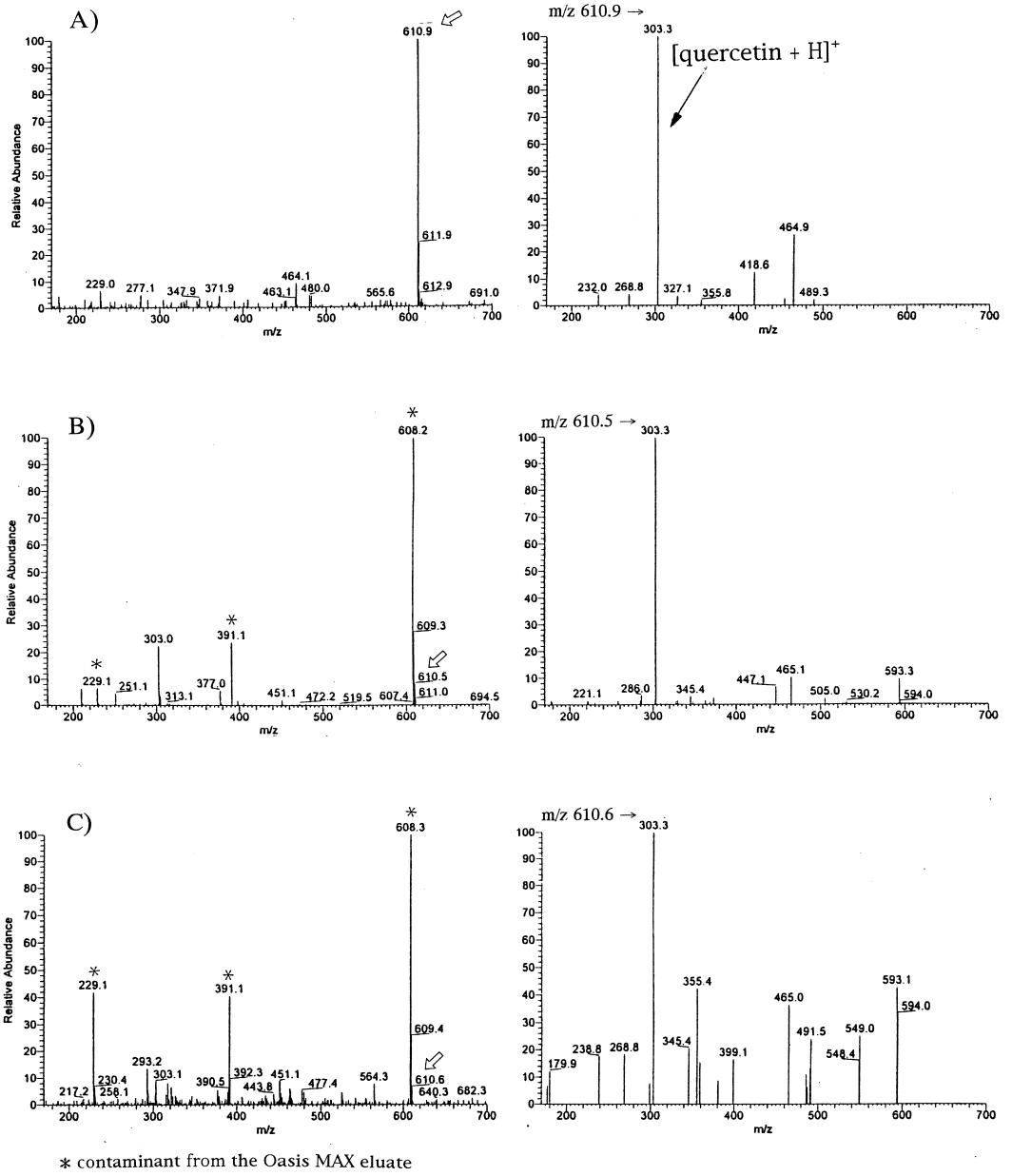

Fig. 4. Positive ion ESI mass spectra (left: MS spectra, right: MS–MS spectra) of rutin. (A) Authentic standard (40 ng on-column); (B) eluate by the Oasis MAX cartridge treatment of aqueous rutin sample (40 ng of rutin in 3 ml of water); (C) plasma extract (5 h after oral administration).

rutin (610 u plus 1). The MS–MS analysis of the *m*/*z* 611 peak as precursor ion gave a base peak at *m*/*z* 303 (quercetin, 302 u plus 1) (Fig. 4A, right). Fig. 4B (left) shows the positive ion ESI mass spectrum of a sample containing rutin. The sample was prepared by adding 40 ng to 3 ml of water and then extracting with an Oasis MAX cartridge as described in the Experimental section. The ions at *m*/*z* 608, 391 and 229 appeared also in the eluate from a blank water sample (without rutin) upon

treating with the Oasis MAX cartridge. When the ion at m/z 611 was subjected to the MS–MS analysis as precursor ion, the quercetin ion at m/z 303 was observed (Fig. 4B, right). LC–ESI-MS spectra of a peak corresponding to rutin on the HPLC chromatogram of a human plasma sample obtained 5 h after oral administration of rutin are shown in Fig. 4C (left: MS spectrum, right: MS–MS spectrum). The appearance of the quercetin ion at m/z 303 in the MS–MS spectrum as observed in Fig. 4A and B strongly indicates the presence of rutin in the human plasma sample.

A time course of plasma concentrations of rutin measured by HPLC is shown in Fig. 5. Rutin was not detected in the blood sample collected just before the intake of 500 mg rutin. There was a relatively slow rise in the plasma concentration of rutin to 2.6 ng/ml at the first blood sampling time of 2 h after the oral administration, giving a peak level of 63.22 ng/ml at 5 h. From the decline phase of semilogarithmic plots of the plasma concentration versus time, the elimination half-life ($t_{1/2}$) of rutin was calculated to be approximately 2.5 h. When the same subject orally received 500 mg of rutin on a different day, a lower peak level of 28.10 ng/ml was observed 5 h after the intake of rutin. It is then possible that the gastrointestinal absorption of rutin may vary to a large extent among the days and/or the subjects in different time occasions as in our previous observation for the absorption of naringin (naringenin-7-rhamnoglucoside) [29].

The present method provides a sensitive and reliable technique for the determination of plasma concentration of rutin. This method will be applicable for rutin level monitoring during pharmacokinetic studies because of the easy sample preparation and the utilization of commonly available chromatographic instruments.

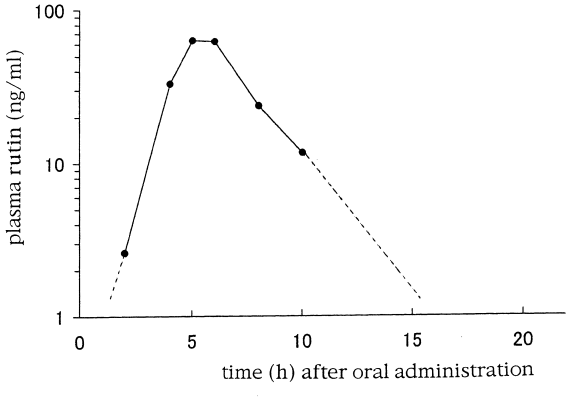

Fig. 5. Plasma concentration of rutin (ng/ml) after intake of rutin (500 mg) by a healthy volunteer.

Acknowledgements

The authors thank Mr. T. Tamura of Kyorin University for his technical assistance.

References

[1] C.A. Rice-Evans, N.J. Miller, G. Paganga, Free Radic. Biol. Med. 20 (1996) 933.
[2] S.V. Jovanovic, S. Steenken, M. Tosic, B. Marjanovic, M.G. Simic, J. Am. Chem. Soc. 116 (1994) 4846.
[3] G. Cao, E. Sofic, R.L. Prior, Free Radic. Biol. Med. 22 (1997) 749.
[4] G. Paganga, N. Miller, C.A. Rice-Evans, Free Radic. Res. 30 (1999) 153.
[5] R.L. Singhal, Y.A. Yeh, N. Prajda, E. Olah, G.W. Sledge Jr., G. Weber, Biochem. Biophys. Res. Commun. 208 (1995) 425.
[6] F.V. So, N. Guthrie, A.F. Chambers, M. Moussa, K.K. Carroll, Nutr. Cancer 26 (1996) 167.
[7] L.G. Menon, R. Kuttan, M.G. Nair, Y.-C. Chang, G. Kuttan, Nutr. Cancer 30 (1998) 74.
[8] M. Messina, S. Barnes, K.D. Setchell, Lancet 350 (1997) 971.
[9] J.V. Formica, W. Regelson, Food Chem. Toxicol. 33 (1995) 1061.
[10] G. Paganga, C.A. Rice-Evans, FEBS Lett. 401 (1997) 78.
[11] P.C.H. Hollman, J.M.P. van Trijp, M.N.C.P. Buysman, M.S.v.d. Gaag, M.J.B. Mengelers, J.H.M. de Vries, M.B. Katan, FEBS Lett. 418 (1997) 152.
[12] K. Herrmann, Z. Lebensm. Unters. Forsch. 186 (1988) 1.
[13] K. Herrmann, J. Food Technol. 11 (1976) 433.
[14] B. Jeffrey, F.R.S. Harborne, B. Herbert, in: The Handbook of Natural Flavonoids I, Wiley, 1999, p. 367.
[15] J. Budzianowski, K. Korzeniowska, E. Chmara, A. Mrozikiewicz, Phytother. Res. 13 (1999) 166.
[16] A. Nègre-Salvayre, A. Affany, C. Hariton, R. Salvayre, Pharmacology 42 (1991) 262.
[17] G. Achilli, G. Cellerino, P.H. Gamache, G. Melzi d'Eril, J. Chromatogr. 632 (1993) 111.
[18] M.G.L. Hertog, P.C.H. Hollman, B. van de Putte, J. Agric. Food Chem. 41 (1993) 1242.
[19] A. Crozier, M.E.J. Lean, M.S. McDonald, C. Black, J. Agric. Food Chem. 45 (1997) 590.

[20] A. Escarpa, M.C. González, J. Chromatogr. A 823 (1998) 331.
[21] P. Pietta, A. Bruno, P. Mauri, A. Rava, J. Chromatogr. 593 (1992) 165.
[22] B. Liu, D. Anderson, D.R. Ferry, L.W. Seymour, P.G. de Takats, D.J. Kerr, J. Chromatogr. B 666 (1995) 149.
[23] J.G. Supko, L.R. Phillips, J. Chromatogr. B 666 (1995) 157.
[24] H. Tsuchiya, J. Chromatogr. B 720 (1998) 225.
[25] P.L. Mauri, L. Iemoli, C. Gardana, P. Riso, P. Simonetti, M. Porrini, P.G. Pietta, Rapid Commun. Mass Spectrom. 13 (1999) 924.
[26] K. Ishii, S. Urano, T. Furuta, Y. Kasuya, J. Chromatogr. B 655 (1994) 300.
[27] K. Ishii, T. Furuta, Y. Kasuya, J. Chromatogr. B 683 (1996) 225.
[28] K. Ishii, T. Furuta, Y. Kasuya, J. Chromatogr. B 704 (1997) 229.
[29] K. Ishii, T. Furuta, K. kasuya, J. Agric. Food Chem. 48 (2000) 56.
[30] D.J.L. Jones, C.K. Lim, D.R. Ferry, A. Gescher, Biomed. Chromatogr. 12 (1998) 232.
[31] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGiveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.