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Intestinal absorption of luteolin and luteolin 7-*O*-β-glucoside in rats and humans

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Abstract In this study, we investigated the intestinal absorption of luteolin and luteolin 7-*O*-β-glucoside in rats by HPLC. The absorption analysis using rat everted small intestine demonstrated that luteolin was converted to glucuronides during passing through the intestinal mucosa and that luteolin 7-*O*-β-glucoside was absorbed after hydrolysis to luteolin. Free luteolin, its conjugates and methylated conjugates were present in rat plasma after dosing. This suggests that some luteolin can escape the intestinal conjugation and the hepatic sulfation/methylation. LC/MS analysis showed that the main conjugate which circulates in the blood was a monoglucuronide of the unchanged aglycone. Luteolin in propyleneglycol was absorbed more rapidly than that in 0.5% carboxymethyl cellulose. The plasma concentration of luteolin and its conjugates reached the highest level 15 min and 30 min after dosing with luteolin in propyleneglycol, respectively. HPLC analysis also allowed us to demonstrate the presence of free luteolin and its monoglucuronide in human serum after ingestion of luteolin.

Key words: Luteolin; Luteolin 7-*O*-β-Glucoside; Intestinal absorption; Glucuronidation; Plasma

1. Introduction

Flavonoids occur naturally in the plant kingdom. They are common dietary components of vegetables, fruits, wine and tea. Epidemiological studies showed that the dietary intake of flavonoids was inversely associated with the mortality from coronary heart disease [1] and the incidence of stroke [2]. These correlations may implicate in part the inhibition of low density lipoprotein oxidation and platelet aggregation [3,4]. Flavonoids have been reported to exert an antioxidant activity due to their ability to scavenge free radicals or to chelate metal ions [5,6].

Luteolin, the flavone subclass of flavonoids, usually occurs as glycosylated forms in celery, green pepper, perilla leaf and camomile tea, etc., and as an aglycone in perilla seeds. It has been reported to be non-mutagenic [7], antimutagenic [8], antitumorigenic [9] and anti-inflammatory-allergic [10] and has been recognized as a hydroxyl radical scavenger [5] and an inhibitor of protein kinase C [11]. Our previous study demonstrated that preadministration of luteolin reduced the frequency of micronucleated reticulocytes (MNRETs) in mouse peripheral blood cells [12], suppressed γ-ray-induced lipid peroxidation in mouse bone marrow and spleen [13] and also inhibited the doxorubicin-induced elevation of the lipid peroxide level in mouse heart and bone marrow [14].

It is very important to evaluate the bioavailability of flavonoids in order to clarify whether the absorbed flavonoids function as antioxidants in vivo. Recently, Hollman et al. reported that humans absorb appreciable amounts of quercetin and the absorption is enhanced by conjugation with glucose [15]. Manach et al. demonstrated that dietary rutin was recovered in substantial concentration in rat plasma as two conjugated metabolites and was absorbed more slowly than quercetin [16]. Rutin must be hydrolyzed by the intestinal microflora. Liu et al. characterized the metabolites of luteolin in rat urine and bile by GC/MS analysis [17]. However, there is little information concerning pharmacokinetics and identification of metabolites in the blood plasma after ingestion of flavonoids. The mechanism of intestinal absorption and the pharmacokinetics of luteolin and its glycosides have not been well elucidated so far.

In this study, we investigated how luteolin and luteolin 7-*O*-β-glucoside are absorbed from the digestive tract using rat everted intestine, determined the plasma levels of luteolin and its conjugates by HPLC and LC/MS analyses, and compared the metabolites in rat plasma with those in human plasma.

2. Materials and methods

2.1. Chemicals

Luteolin and chrysoeryol were isolated from perilla seed and purified by HPLC at the Oryza Oil and Fat Chemical Co. (Ichinomiya, Japan). Luteolin 7-*O*-β-glucoside and diosmetin were obtained from Extrasynthese (Genay, France). β-Glucuronidase/sulfatase and β-glucuronidase were purchased from Sigma (St. Louis, MO, USA) and Wako Pure Chem. Ind. (Osaka, Japan), respectively.

2.2. Animals and diets

Male SD rats (7–9 weeks old, SLC, Hamamatsu, Japan), weighing 180–200 g, were housed in an air-conditioned room, fed CE-2 commercial food pellets (Crea Japan, Tokyo, Japan) ad libitum at first, and this diet was changed to a synthetic basic diet which consists of 38% corn starch, 25% casein, 10% α starch, 8% cellulose powder, 6% minerals, 5% sugar, 2% vitamins and 6% lard (Oriental Yeast, Tokyo, Japan) one week before the experiments. Three to five rats were assigned to each experimental group. Animals were maintained and handled according to Guidelines for the Regulation of Animal Experimentation Committee of the University of Shizuoka.

2.3. Sample preparation of blood

Rats fasted overnight were anesthetized with ethyl ether at different times after administration of luteolin or luteolin 7-*O*-β-glucoside (50 μmol/kg in 0.5% carboxymethyl cellulose sodium, CMC-Na or propyleneglycol) by gastric intubation, and blood was withdrawn from the abdominal aorta into heparinized tubes. Two human volunteers (female: 55 kg, male: 75 kg) gave informed consent before the experi-

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ment. They ingested 50 mg of luteolin suspended in starch solution and their venous blood was collected in vacuum containers by a medical doctor 3 h after ingestion.

2.4. Absorption experiment with everted intestine

The intestinal absorption of luteolin and luteolin 7-*O*-β-glucoside from the mucosa to the serosal side was studied as previously described [18] with some modifications. Briefly, rats fasted overnight were anesthetized with pentobarbital, and six consecutive segments of 2.5 cm each were dissected from the middle part of the upper half (jejunum) of the small intestine and rinsed with Ringer's solution which contained 140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM KH_2PO_4, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM $MgCl_2{\cdot}6H_2O$, and 1 mM glucose (pH 7.4). They were everted and fixed over a fenestrated conical polypropylene tube attached with a Tygon tubing. These sacs were placed in 5 ml of Ringer's solution containing 5 mM sodium cholate, 1.9% Tween 20 and 1 mM luteolin or luteolin 7-*O*-β-glucoside, and the serosal side was filled with 0.3 ml of Ringer's solution. They were incubated bubbling with 95% O_2/5% CO_2 at 37°C. The medium of the serosal side was substituted with fresh Ringer's solution 15 min after the start of incubation.

2.5. HPLC analysis

HPLC analysis was performed by the method described previously with some modification [19]. The plasma (0.5 ml) and the incubation medium of the serosal side were acidified with the same volume of 0.01 M oxalic acid.

This solution was applied to a Sep-Pak C_{18} cartridge. After washing the cartridge with 0.01 M oxalic acid, methanol/water/0.5 M oxalic acid (25:73:2, v/v) and distilled water, the methanol eluate was obtained. For samples in Fig. 5, the cartridge was not washed with methanol/water/0.5 M oxalic acid (25:73:2, v/v/v). The eluate was evaporated to dryness and the residue was dissolved in 100 μl of methanol. After centrifugation for 2 min at 0°C at $20\,000\times g$, the supernatants were analyzed chromatographically by a JASCO HPLC system (Tokyo, Japan) using a Capcell Pak C_{18}-UG120 column (150×4.6 mm I.D., Shiseido, Tokyo, Japan) and UV detection (349 nm). The mobile phase contained the following: Solvent A, methanol with 0.03% trifluoroacetic acid; Solvent B: distilled water with 0.03% trifluoroacetic acid. The column temperature was maintained at 50°C and the flow rate was 0.7 ml/min. Quantification of luteolin was done by measuring the peak areas based on calibration plots of the peak area of standard luteolin at various concentrations.

For detection of the conjugates, each sample was acidified with 1 M acetate buffer (pH 4.5) and was preincubated for 2 min at 37°C. Solutions were treated with 5.4×10^2 units/ml β-glucuronidase and 0.2×10^2 units/ml sulfatase for 20 min at 37°C, and then the same volume of 0.01 M oxalic acid was added. The mixtures were centrifuged for 5 min at 8000 rpm. Supernatants were prepared in the same way as described above.

The recoveries of luteolin from plasma and the incubation medium of the serosal side were obtained by spiking luteolin at 1 mg/ml into each sample. Each recovery was 103.8 ± 2.4% ($n=5$), 95.3 ± 7.7% ($n=5$), respectively.

2.6. LC-MS analysis

LC-MS (FRIT FAB ionization method, negative mode) analysis was performed in a JEOL JMS DX-303 mass spectrometer with a JASCO liquid chromatograph PU-980 apparatus. Matrix solution (1% glycerol in methanol) was mixed after the UV detector. The LC conditions were as follows: column, Capcell Pak C_{18}-UG120 column (150×2.0 mm I.D., Shiseido, Tokyo, Japan); detection, UV 349 nm; flow rate, 163 μl/min; mobile phase, the same as in HPLC analysis except gradient conditions. The analysis was carried out at 40°C.

3. Results

Fig. 1A,B,C shows the HPLC chromatograms of the standards (luteolin 7-*O*-β-glucoside, luteolin) and the constituents appearing in the serosal side after intestinal absorption of luteolin or luteolin 7-*O*-β-glucoside. Two metabolite peaks (a: 13.1 min; b: 20.0 min) and luteolin peak (c: 22.8 min) were observed. After treatment with β-glucuronidase/sulfatase, peaks a and b disappeared and peak c increased (Fig. 1B). These results demonstrated that peaks a and b were glucuro- and/or sulfo-conjugates. After intestinal absorption of luteolin 7-*O*-β-glucoside, peaks a, b and d were observed. Peak d at a retention time of 12.6 min nearly corresponded with that of standard luteolin 7-*O*-β-glucoside. Because β-glucuronidase/sulfatase was shown to possess β-glucosidase activity, β-glucuronidase was used for deconjugation in the case of luteolin 7-*O*-β-glucoside. After treatment with this enzyme, peaks a and b disappeared and the luteolin peak (c) increased as observed in the case of luteolin (Fig. 1C). These results suggest that luteolin 7-*O*-β-glucoside is mainly hydrolyzed to luteolin and then luteolin is absorbed, and that peaks a and b are glucuronides.

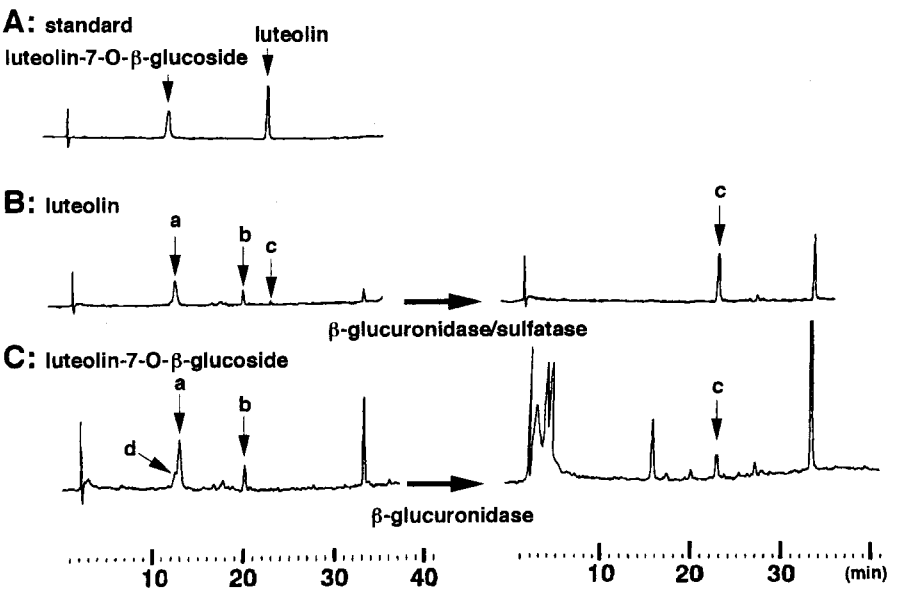

Fig. 1. HPLC chromatograms of the constituents appearing in the serosal side before and after β-glucuronidase or β-glucuronidase/sulfatase treatment. Gradient conditions: A/B = 30/70, 0–5 min; 30/70 → 65/35, 5–30 min; 65/35, 30–40 min; 65/35 → 100/0, 40–45 min. Peaks a: 13.1 min; b: 20.0 min; c: 22.8 min (luteolin); d: 12.6 min; luteolin 7-*O*-β-glucoside: 12.8 min.

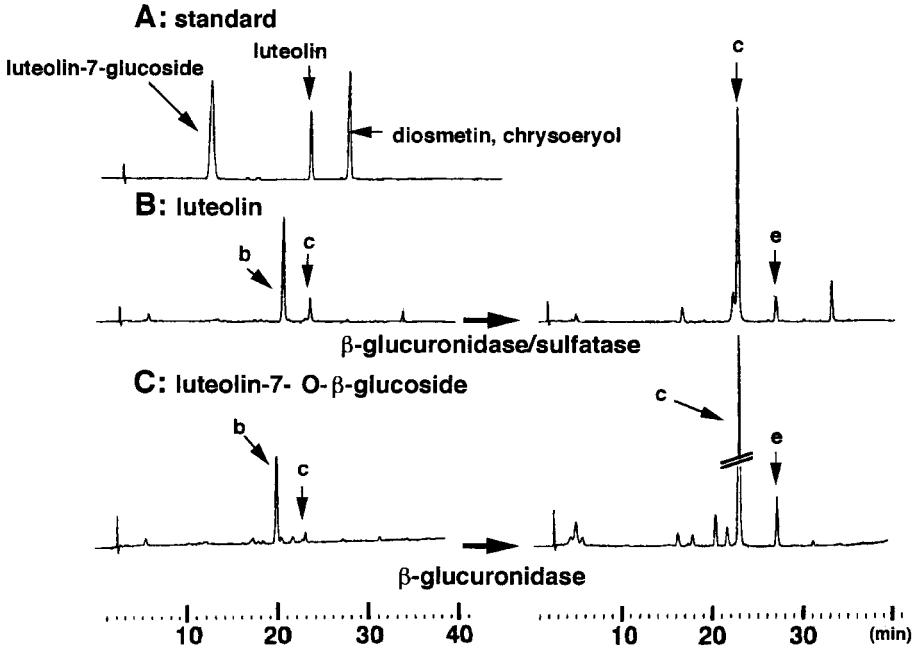

Fig. 2. HPLC chromatograms of plasma extracts from luteolin or luteolin 7-*O*-β-glucoside administered rats before and after β-glucuronidase or β-glucuronidase/sulfatase treatment. Gradient conditions and peaks b and c: see Fig. 1. Peak e: 26.9 min (diosmetin and chrysoeryol).

The HPLC elution profiles of rat plasma 3 h after administration of luteolin and luteolin 7-*O*-β-glucoside (50 μmol/kg in 0.5% CMC-Na) by gastric intubation are shown in Fig. 2B and C. The same two peaks (b, c) were observed as in Fig. 1B and C, but peak a was not detected. After treatment with β-glucuronidase/sulfatase, the peaks of luteolin (c) and diosmetin/chrysoeryol (e: 26.9 min) increased. Free luteolin, luteolin conjugates and methylated conjugates were present in rat plasma. However, luteolin 7-*O*-β-glucoside was not detected in rat plasma.

The methanol fraction of the plasma extract from rats administered luteolin was analyzed by LC-MS. Fig. 3A,B,C shows the HPLC chromatogram and mass spectra. Peaks b′ and c′ in the HPLC chromatogram corresponded to peaks b and c in Figs. 1 and 2. The negative FAB-MS of peaks b′ and c′ showed molecular ion peaks at *m/z* 461 and 285[M-H]$^-$ corresponding to a monoglucuronide of luteolin and luteolin, respectively. These results indicate that free luteolin is present in rat plasma and that the main conjugate is a monoglucuronide of unchanged luteolin.

In each experiment mentioned above, 3–5 rats were used and the same results were obtained. Therefore, representative results are shown in Figs. 1–3.

The time courses of the concentration of luteolin and luteolin conjugates in rat plasma after administration of luteolin in 0.5% CMC-Na and propyleneglycol by gastric intubation (50 μmol/kg) are shown in Fig. 4. The amount of luteolin conjugates in rat plasma was obtained by subtracting the amount of free luteolin from the total amount of luteolin after treatment with β-glucuronidase/sulfatase. The concentration of luteolin and its conjugates in rat plasma increased to the highest level 15 min (3.08 nmol/ml) and 30 min (14.1 nmol/ml) after administration of luteolin in propyleneglycol and decreased gradually. The intestinal absorption was faster and the plasma concentration of both luteolin and its conjugates was three times higher in rats administered luteolin in propyleneglycol than in those administered luteolin in 0.5% CMC-Na.

As shown in Fig. 5, peaks b″ and c″, of which the retention time corresponded to those of peaks b (luteolin monoglucuronide) and c (luteolin) in Figs. 1 and 2, were detected in human serum as well as in rat plasma.

4. Discussion

Recently, the bioavailability, pharmacokinetics and metabolism of flavonoids have been focused on in order to evaluate their important role in the chemoprevention of diseases such as cancer, heart disease and diabetes, etc. Flavonoids usually occur in dietary plants in the glycosylated form. Hollman et al. have reported that quercetin glycosides from onion were absorbed more efficiently than the aglycone form in ileostomy patients [15]. Paganga and Rice-Evans have shown evidence for the absorption of flavonoids and their presence in human plasma in the glycosylated form [20]. Whether the glycosides can be absorbed or not and how flavonoids pass through the intestinal mucosa, however, has not been elucidated.

In this study, the experiment with rat everted small intestine demonstrated that luteolin 7-*O*-β-glucoside was hardly absorbed by itself and luteolin glucuronides were detected after intestinal absorption. Further, luteolin 7-*O*-β-glucoside was not detected in rat plasma after administration. It has been reported that the intestinal microbacteria can hydrolyze glucosides of flavonoids and produce aglycones [21]. Therefore, we suggested that luteolin 7-*O*-β-glucoside may be first hydrolyzed to luteolin by the microbacteria which were present on the surface of the intestinal mucosa. However, we have previously reported that αG-Rutin (4^G-α-D-glucopyranosylrutin), which is a water-soluble flavonoid, was absorbed into the circulation very rapidly and the metabolite was present in the glycosylated form in mouse plasma [19]. It may depend

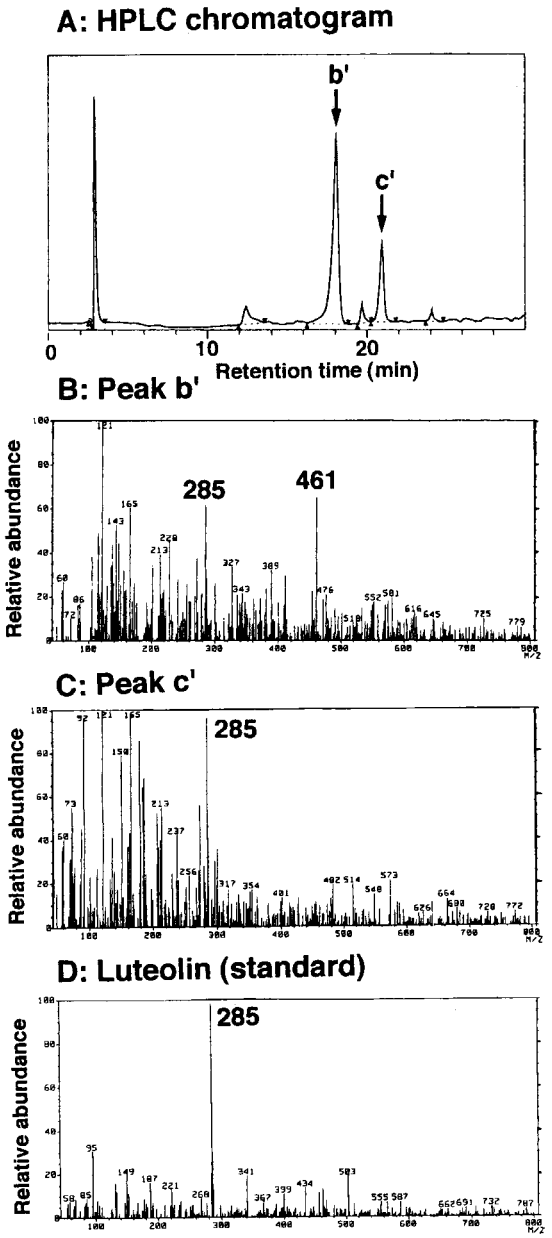

Fig. 3. HPLC chromatogram and mass spectra of plasma extracts from luteolin administered rats and standard luteolin. Gradient conditions: A/B = 30/70 → 65/35, 0–26 min; 65/35 → 100/0, 26–36 min; 100/0 → 30/70, 42–60 min. Peaks b′: 18.1 min; c′: 20.9 min (luteolin).

on the type of glycosylated forms whether glycosides can be absorbed or not. Water-soluble flavonoids may be absorbed without hydrolysis by intestinal microbacteria.

On the other hand, we showed that the plasma of rats administered luteolin orally contained free luteolin (unmodified form), glucuronide or sulfate-conjugates of unchanged luteolin and *o*-methyl luteolin (diosmetin or chrysoeryol). A number of previous studies have reported that no free form of quercetin or diosmetin was detected in rat plasma [22,23]. However, free luteolin was present in rat plasma. Some luteolin could escape the intestinal conjugation and hepatic sulfation/methylation. The experiment with rat everted small intestine also showed that absorbed luteolin was converted to glucuronides passing through the intestinal mucosa. Though the liver is generally considered to have the capacity to conjugate flavonoids, the glucuronidation occurs rapidly by UDP-glucuronosyltransferase in the intestinal mucosa. Further we found that luteolin was absorbed more efficiently from the duodeno-jejunum than the ileum (data not shown).

Pharmacokinetic profiles of free luteolin and luteolin conjugates in rat plasma are shown in Fig. 4. When rats were given luteolin in propyleneglycol (50 μmol/kg) orally, the total luteolin concentration in rat plasma 30 min after dosing was 15.5 ± 3.8 nmol/ml. Diosmetin was shown to circulate in the blood in the glucuronide form, and its plasma concentration at a high level was ∼10 μg/ml (∼33 nmol/ml) after oral administration (100 mg/kg) [23]. King et al. have examined the pharmacokinetics of the soy isoflavone genistein following a single oral dose of genistein (20 mg/kg) [24]. After treatment with β-glucuronidase, the plasma concentration of genistein at 2 h after dosing was 11.0 ± 2.3 nmol/ml, which was similar to our results. On the other hand, the excretory recovery for 24 h as unmodified luteolin from the urine was about 4%.

LC/MS analysis showed that the main metabolite in plasma was a monoglucuronide. Luteolin possesses four hydroxyl groups at the 3′-, 4′-, 5-, and 7-positions which are available for glucuronidation. It is unclear which position of luteolin is a glucuronidation site at the present time. The biological activity of luteolin is considered to be associated in part with its antioxidant potential. The presence of hydroxyl groups is very important for antioxidant activity. It was suggested that the •OH scavenging activity is directly related to the number of hydroxyl groups substituted on ring B, especially at the 3′-position [5]. Cao et al. have reported that dihydroxyl groups at the 3′- and 4′-positions play an important role in antioxidant reaction. Antioxidant activity decreases with methylation of the hydroxyl group [25]. Between the hydroxyl group at the 5-position and the neighboring ketone at the 4-position, or the ortho-dihydroxyl groups at the 3′- and 4′-positions, chelating complexes with metal ions are formed. Therefore, luteolin monoglucuronide possesses chelating ability. The antioxidant activity of luteolin monoglucuronide might be lower than that of luteolin. However, it does not lose antioxidant potential completely.

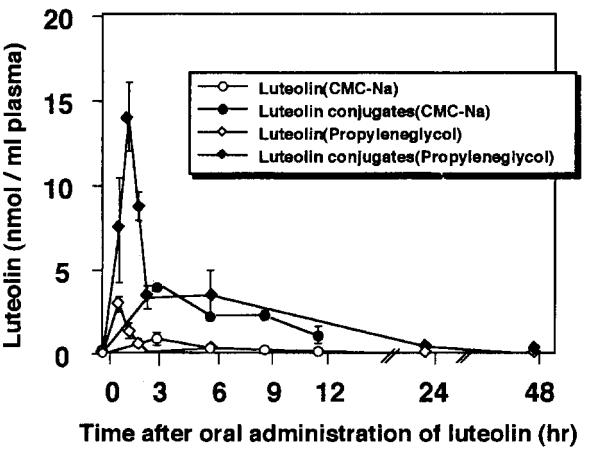

Fig. 4. Concentration of luteolin and its conjugates in rat plasma after oral administration of luteolin. Luteolin: 50 μmol/kg (0.5% CMC-Na and propyleneglycol); luteolin conjugates: plasma samples were treated with β-glucuronidase/sulfatase. Each value represents the mean ± S.E. ($n = 3$).

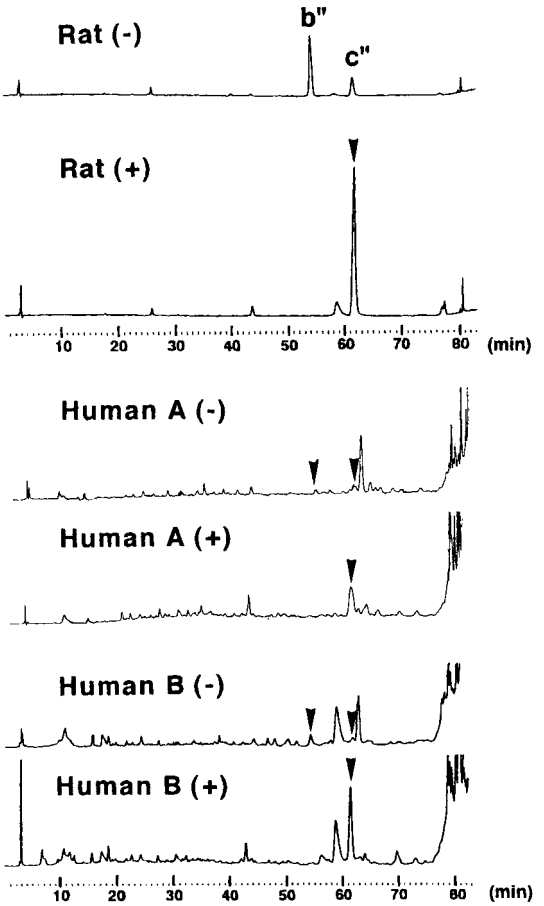

Fig. 5. HPLC chromatograms of extracts from rat plasma and human serum 3 h after oral administration of luteolin. Luteolin: rats (50 μmol/kg in 0.5% CMC-Na); humans (A: male; B: female; 50 mg in starch solution). Gradient conditions: A/B = 5/95, 0–5 min; 5/95→30/70, 5–30 min; 30/70→40/60, 30–70 min; 40/60→100/0, 70–80 min. Peaks b″: 54.2 min (luteolin monoglucuronide); c″: 61.5 min (luteolin). (+), (−): with and without β-glucuronidase/sulfatase.

It is noteworthy that free luteolin and its monoglucuronide are present in human serum after dosing. According to Manach et al., the high plasma concentration of quercetin metabolites was maintained with a regular supply of quercetin in the diet [16]. Therefore, it is suggested that maintenance of the flavonoid level in the blood might improve the antioxidant status in vivo. On the other hand, the pharmacological activity of glucuronides such as morphine glucuronide was shown recently [26]. To clarify that luteolin monoglucuronides demonstrate pharmacological activity, further investigations are required.

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