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Method for Analysis of Tannic Acid and Its Metabolites in Biological Samples: Application to Tannic Acid Metabolism in the Rat

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A new analytical method for measuring tannic acid (TA) using tannase was developed and applied to the investigation of TA metabolism in the rat following oral administration at a dose of 1.0 g/kg. The proposed method for TA determination was based on the enzymatic hydrolysis of TA to gallic acid (GA) and subsequent determination by HPLC. TA metabolites were determined by HPLC. 4-*O*-Methylgallic acid (4-OMGA), pyrogallol (PY), and resorcinol (RE) were detected in serum. TA was excreted into urine as GA (0.01%), 4-OMGA (0.10%), PY (0.24%), and RE (2.06%) and into feces as TA (62.74%), GA (0.19%), PY (0.02%), and RE (0.76%) within 54 h after oral administration. It was suggested that >60% of TA remained unchanged but that some was hydrolyzed to GA by tannase in the intestine and further metabolized to 4-OMGA, PY, and RE.

KEYWORDS: **Tannic acid; tannase; metabolism; gallic acid; 4-*O*-methylgallic acid; pyrogallol; resorcinol; rat**

INTRODUCTION

Tannic acid (TA) is a typical hydrolyzed tannin, a mixed gallotannin composed of gallic acid (GA) esters of glucose (*1*−*3*) as shown in **Figure 1**.

TA has several toxic properties in vivo. It suppresses growth (*4*, *5*), binds to proline-rich proteins (*6*), increases fecal excretions of water, lipids, and nitrogen (*7*), inhibits protein absorption from the intestinal tract (*8*), inhibits some intestinal microbial enzymes (*9*), and induces methohemoglobinemia (*10*) when administered orally and damages the abomasum, liver, and kidney in sheep when administered by intra-abomasal dosing (*11*). On the other hand, beneficial effects of TA have recently been reported. It has antimutagenic, anticancer, and antioxidant properties (*12*, *13*), reduces serum cholesterol and triglycerides (*14*−*16*), and suppresses lipogenesis by insulin (*17*).

Metabolism of TA has been investigated in sheep (*9*−*11*, *18*), rat (*7*), mice (*10*), and chicken (*19*). Several studies (*19*−*21*) have reported that GA, 4-*O*-methylgallic acid (4-OMGA), pyrogallol (PY), resorcinol (RE), and ellagic acid (EA) are the metabolites of TA (**Figure 2**) and that some are degraded and enter the TCA cycle. We are not aware of any publications that analyze the binding of TA in biological samples due to the difficulty of determining TA in vivo. Thus, the overall metabolism of TA is still incompletely understood.

Here we report a new analytical method for measuring TA as GA by HPLC after hydrolysis with tannase. Using this method, we investigated the metabolism of TA in the rat including binding of TA in feces.

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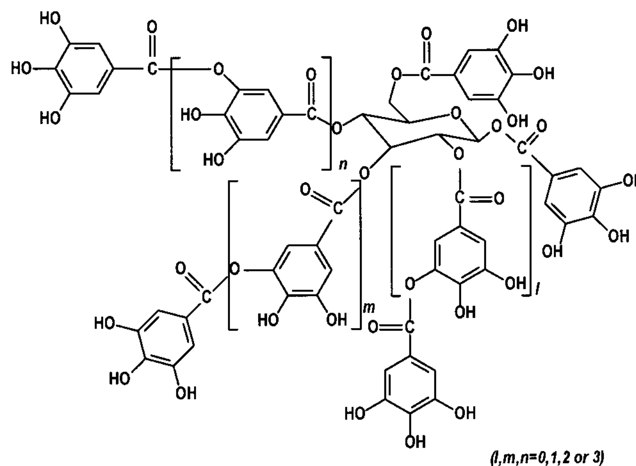

Figure 1. Chemical structure of TA.

MATERIALS AND METHODS

Materials and Instruments. Gallic acid (3,4,5-trihydroxybenzoic acid, GA), resorcinol (1,3-benzenediol, RE), phloroglucinol (1,3,5-benzenetriol, PH), ellagic acid (2,3,7,8-tetrahydoxyl[1]benzopyrano-[5,4,3-*cde*][1]benzopyran-5,10-dione, EA), and flavone (2-phenyl-4*H*-1-benzopyran-4-one) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-*O*-Methylgallic acid (3,5-dihydroxy-4-methoxybenzoic acid, 4-OMGA) and 3-*O*-methylgallic acid (3,4-dihydroxy-5-methoxybenzoic acid, 3-OMGA) were purchased from

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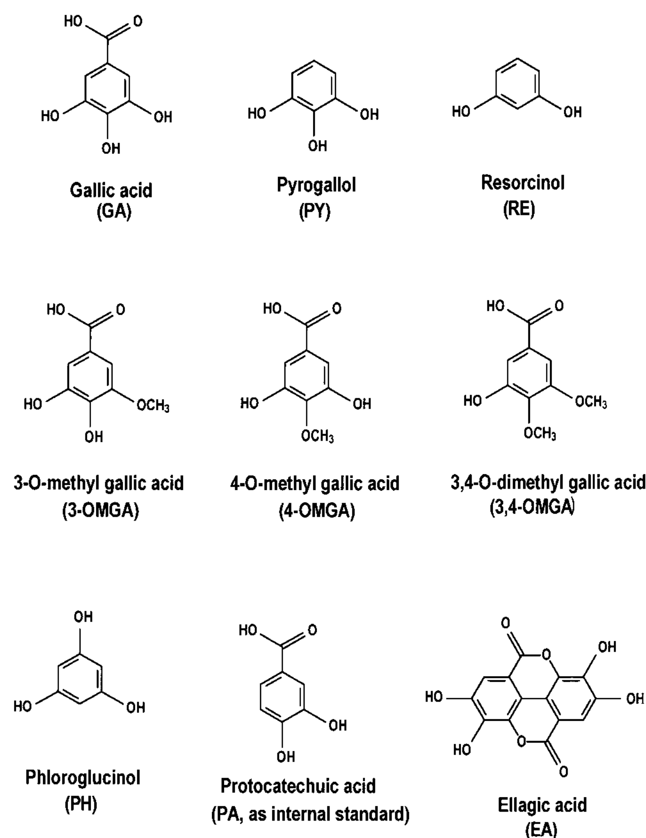

Figure 2. Chemical structures of the metabolites of TA and their related compounds.

Apin Chemicals Ltd. (Abingdon, U.K.), 3,4-*O*-dimethylgallic acid (3-hydroxy-4,5-dimethoxybenzoic acid, 3,4-OMGA) was from Sigma Chemical Co. (St. Louis, MO), and protocatechuic acid (3,4-dihydroxybenzoic acid, PA) was from Aldrich Chemical Co., Inc. (Milwaukee, WI). (+)-Catechin (CT), (−)-epicatechin (EC), (−)-catechin gallate (CG), (−)-epicatechin gallate (EGC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG), and Polyphenon-100 (green tea polyphenol) were purchased from Funakoshi (Tokyo, Japan). 1,2,3,6-Tetra-*O*-galloyl-*β*-D-glucose (TGG) and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (PGG) were kindly provided by Dr. T. Yoshida (Faculty of Pharmaceutical Sciences, Okayama University). Ion-exchanged and redistilled water was used throughout the experiments. HPLC grade acetonitrile, ethyl acetate, *n*-hexane, and methanol were used for analyses. Other reagents were also of analytical grade.

Tannic acid (TA) for chemical analysis was purchased from Wako Pure Chemical Industries and used for the experiments. Two kinds of food additive grade TA (no. 1 and 2) were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and Fuji Chemical Industry, Co., Ltd. (Wakayama, Japan), respectively. Tannase (EC 3.1.1.20, from *Aspergillus orizae*, 50000 units) was purchased from Wako Pure Chemical Industries; *β*-glucuronidase (EC 3.2.1.31 from *Escherichia coli*, type X-A, 100000 units) and sulfatase (EC 3.1.6.1 from *Abalone entrails*, type VIII, 1000 units) were from Sigma Chemical Co. The HPLC columns STR ODS-II (4.6 mm i.d. × 250 mm) and Develosil 300 ODS-HG-5 (4.6 mm i.d. × 250 mm) were purchased from Shinwa Chemical Industries, Ltd. (Kyoto, Japan) and Nomura Chemical Co., Ltd. (Seto, Japan), respectively.

A Shimadzu HPLC apparatus, Class-VP series (Kyoto, Japan), equipped with system controller SCL-10AVP, pump LC-10ADVP, degasser DGU-14A, autoinjector SIL-10ADVP, column oven CTO-10AC, and diode array detector SPD-M10AVP, was used to analyze TA and its metabolites.

Animal Experiments. All procedures involving animals were conducted according to Japanese law (Bulletin of the Prime Minister's Office, no. 6, March 1980) and guidelines established by the National Institute of Health Sciences. Male Wistar rats (4 weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan) and kept in an air-conditioned room (23 ± 1 °C, 50−60% humidity) illuminated for 12 h a day (7:00 a.m. to 7:00 p.m.). Rats weighing 252−285 g were used in the experiments. Each group consisted of five or six rats. Rats had free access to a normal F-2 diet (Funahashi Farm, Chiba, Japan) and water throughout the experiments.

No TA or GA was detected in the F-2 diet. The content of GA in TA used in this study and in food additive grade TA 1 and 2 was <0.3% (w/w) (n = 5−10, determined by HPLC). The same lot of TA was used throughout the study. The TA solution used for oral administration was freshly prepared before use by dissolving 1.0 g of TA in water and bringing the volume to 10 mL. The TA solution was administered to rats at a dose of 10 mL/kg of body weight between 1:00 p.m. and 3:00 p.m.

In experiment 1, TA was administered orally at a dose 1.0 g/kg. Rats were anesthetized with diethyl ether, and blood was collected by heart puncture at 0.5, 1, 1.5, 2, 4, 5, 6, 17, and 29 h after oral administration. Serum was obtained by centrifugation at 1500 rpm for 15 min at an ambient temperature and stored below −20 °C until analysis.

In experiment 2, TA was administered orally at a dose of 1.0 g/kg. Urine and feces were collected at 5.5, 24.5, and 54 h and at 24.5 and 54 h after TA administration, respectively. Collected urine was immediately filtered through no. 5A filter paper and adjusted to 50 mL after the addition of 100 mg of ascorbic acid and stored below −20 °C until analysis. Collected feces were dried overnight below 40 °C, ground to a powder, and stored below −20 °C until analysis.

Analytical Methods. *(1) Analysis of TA Metabolites in Serum and Urine.* TA metabolites in serum and urine were analyzed according to the method of Zhu et al. (*22*) with some modifications. To 0.5 mL of serum or 2 mL of urine were added 2 mL of 0.1 M acetate buffer (pH 5.0), 0.1 mL of a 4% ascorbic acid solution (in water), 0.1 mL of a 4% Na_2EDTA solution (in water), and 0.2 mL of a 0.6 M $CaCl_2$ solution (in water). For the determination of total TA metabolites, *β*-glucuronidase (1000 units) and sulfatase (25 units) were added to the serum preparation and incubated at 37 °C for 45 min. Enzymatic hydrolysis was stopped by the addition of 0.5 mL of 1 N HCl. TA metabolites were extracted three times with 5 mL of ethyl acetate after the addition of PA (39.33 nmol) as an internal standard (IS). After evaporation to dryness, the residue was dissolved in 2 mL of methanol for determination by HPLC. Free TA metabolites were analyzed without enzymatic hydrolyses.

(2) Analysis of TA and TA Metabolites in Feces. Fecal TA samples were analyzed in triplicate.

About 0.2 g of dried and ground feces was weighed, and the lipids were extracted with 50 mL of *n*-hexane at ambient temperature for 24 h following sonication for 30 min. The residue was dried, and TA and the TA metabolites therein were then extracted with 50 mL of methanol (24 h at ambient temperature following 30 min of sonication). The methanol extract was adjusted to 50 mL (MeOH-soluble fraction), and 2 mL was evaporated to dryness. TA metabolites were analyzed according to the above-mentioned method.

TA content in the MeOH-soluble fraction was determined as follows. Two milliliters of the MeOH-soluble fraction was taken and evaporated to dryness. Five milliliters of 0.05 M citrate buffer (pH 5.5) containing tannase (200 units) was added, and the mixture was incubated at 30 °C for 2 h. Tannase was inactivated by the addition of 0.5 mL of 1.0 N HCl. The hydrolysate was extracted three times with 5 mL of ethyl acetate after the addition of flavone (23.62 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in 2 mL of methanol for determination by HPLC. For the calculation of TA, 0.05−1.0 mg of TA was added to a test tube and hydrolyzed to GA by the addition of 50 units of tannase in 5 mL of 0.05 M citrate buffer (pH 5.5) at 30 °C for 2 h. The liberated GA from standard TA was extracted three times with 5 mL of ethyl acetate after the addition of flavone (23.62 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in 2 mL of methanol for determination by HPLC. A calibration curve relating TA to GA was made on the basis of the GA liberated from a standard TA. Fecal TA content was calculated

Table 1. Relative Retention Times and Detection Limits of Tannic Acid Metabolites and Their Related Compounds[a]

polyphenol[b]	MW	relative retention time (PA = 1.00) STR ODS-II	Develosil 300 ODS-HG-5	detection limit (S/N = 3) (pmol/mL) STR ODS-II	Develosil 300 ODS-HG-5
GA	170.1	0.71	0.68	106	532
3-OMGA	184.1	1.12	1.18	221	276
4-OMGA	184.1	1.17	1.24	108	539
3,4-OMGA	198.2	1.64	1.91	103	257
PH	126.1	0.61	0.57	542	1084
PY	126.1	0.62	0.64	809	1618
RE	110.1	0.98	0.94	675	1351
EA	338.1	1.70	2.13	80.2	1003
PA (IS)	154.1	1.00[c]	1.00[d]		

[a] Conditions for HPLC were as follows: apparatus, Shimadzu Class VP series [pump LC-10ADVP + degasser DGU-14A + column oven CTO-10ACVP + system controller SCL-10ADVP + autoinjector 10ADVP + diode array detector (DAD) SPD-M10AVP]; column, STR ODS-II or Develosil 300 ODS-HG-5; column oven temperature, 35 °C; mobile phase, (A) water/phosphoric acid 1000:1 (v/v), (B) acetonitrile/phosphoric acid 1000:1 (v/v); gradient program B, 0% (0 min) → 15% (20 min) → 30% (25 min) → 100% (45–61 min) → 0 (62–77 min); detector, DAD; monitoring wavelength, 260 nm for EA and 210 nm for others; injection volume, 10 μL. [b] Abbreviations: GA, gallic acid; 3-OMGA, 3-*O*-methylgallic acid; 4-OMGA, 4-*O*-methylgallic acid; 3,4-*O*-methylgallic acid; PH, phloroglucinol; PY, pyrogallol; RE, resorcinol; EA, ellagic acid; PA, protocatechuic acid. [c] Retention time was 17.80 min. [d] Retention time was 12.98 min.

from the calibration curve and corrected by subtraction of the blank value (no tannase treatment).

Total TA content was analyzed as follows. About 0.2 g of dried and ground feces was weighed, and the lipids were extracted with 50 mL of *n*-hexane at ambient temperature for 24 h following sonication for 30 min. After the *n*-hexane was completely removed, 10 mL of 0.05 M citrate buffer (pH 5.5) containing tannase (2000 units) was added and the mixture was incubated at 30 °C for 20 h. Tannase was inactivated by the addition of 1 mL of 1.0 N HCl. The hydrolysate was extracted three times with 5 mL of ethyl acetate after the addition of flavone (944.9 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in methanol and the volume was adjusted to 50 mL. To complete the hydrolysis of TA to GA, 2 mL of the methanol solution was taken and evaporated to dryness. The hydrolysis was repeated by the addition of 100 units of tannase in 5 mL of 0.05 M citrate buffer (pH 5.5) at 30 °C for 2 h. The hydrolysate was then treated as described for the MeOH-soluble fraction. For the calculation of TA, 0.1−10 mg of TA was hydrolyzed with tannase (2000 units) at 30 °C for 20 h. The liberated GA from standard TA was extracted with ethyl acetate three times after the addition of flavone (944.9 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in methanol and the final volume was adjusted to 50 mL and applied for HPLC determination. A calibration curve relating TA to GA was made on the basis of the GA liberated from standard TA. Fecal TA content was calculated from the calibration curve and corrected by subtraction of the blank value (no tannase treatment).

(3) HPLC. HPLC conditions were as follows: column, STR ODS-II (for serum and urinary TA metabolite analysis) or Develosil 300 ODS-HG-5 (for analysis of fecal TA and its metabolites); column oven temperature, 35 °C; mobile phases, (A) water/phosphoric acid 1000:1 (v/v), (B) acetonitrile/phosphoric acid 1000:1 (v/v); gradient program, (B) 0% (0 min) → 15% (20 min) → 30% (25 min) → 100% (45−61 min) → 0% (62−77 min); detector, DAD; wavelengths monitored, 260 nm for EA and 210 nm for others; injection volume, 10 μL.

Because phosphoric acid itself has an absorbance at 210 nm, the HPLC chromatograms were corrected by subtracting the baseline.

Examination of the Analytical Methods. *(1) Hydrolysis of TA by Tannase.* Hydrolysis of TA by tannase was investigated as a function of time. To 50 mg of TA was added 2500 units of tannase in 0.05 M citrate buffer (pH 5.5) in a total volume of 250 mL at 30 °C. Five milliliters of the incubation mixture was sampled in triplicate at 0.5, 1, 2, 3, 4, 5, 6, and 20 h after the addition of tannase. The liberated GA from TA in the incubation mixture was extracted with ethyl acetate three times after the addition of flavone (23.62 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in 2 mL of methanol for determination by HPLC. HPLC conditions are described under Analytical Methods.

(2) Tannase Reactivity toward Various Polyphenols. Five milliliters of 0.05 M citrate buffer (pH 5.5) and tannase (50 units) were added to the test tubes containing CG, ECG, GC, EGC, GCG, EGCG, EA, TGG, PGG, TA, food additive grade TA 1 and 2, or Polyphenon-100 (135.5−1000 μg each). The mixtures were incubated at 30 °C for 2 h. Tannase was inactivated by the addition of 0.5 mL of 1.0 N HCl. The hydrolysates were extracted three times with 5 mL of ethyl acetate after the addition of flavone (23.62 nmol) as an internal standard. After evaporation to dryness, the residues were dissolved in 2 mL of methanol and the hydrolysates were determined by HPLC. HPLC conditions are described under Analytical Methods.

(3) Recovery Tests. Recovery tests of TA and TA metabolites in serum, urine, and feces were performed in three to five trials using serum, urine, and dried feces of a control rat.

For the recovery tests of TA metabolites, and their related compounds adequate amounts of GA, 3-OMGA, 4-OMGA 3,4-OMGA, PY, RE, PH, and EA were added to 0.5 mL of serum, 2 mL of urine, or 2 mL of the MeOH-soluble fraction of 0.2 g of dried feces. TA metabolites were analyzed according to the above-mentioned method without enzymatic hydrolysis. For the recovery test of TA, 100 or 1000 μg of TA was added to 2 mL of the MeOH-soluble fraction and 0.2 g of dried feces, respectively. Samples were treated as described above, and the recoveries were calculated according to the method described under Analytical Methods.

RESULTS

Analytical Methods. Phosphoric acid rather than acetic acid was used to sharpen the shape of peaks that corresponded to each polyphenol. The retention times of the TA metabolites and related compounds (relative to that of the internal standard PA, which was set at 1.00) and their detection limits are shown in **Table 1**. Each compound was completely separated by both columns. The detection limits using the STR ODS-II column were lower than that observed using the Develosil 300 ODS-HG-5 column.

Prior to measuring TA, a hydrolysis time course was performed (**Figure 3A**). The generated GA reached a maximum value in 2 h with 5.022 nmol of GA produced from 1 μg of TA. The produced GA might be degraded during a longer incubation time, but the degree of degradation of GA seemed to be mild: 4.556 nmol of GA produced from 1 μg of GA after a 20 h incubation. Therefore, the analysis of fecal TA was done using a 2 or 20 h incubation. A calibration curve is shown in **Figure 3B**. The linear range of the curve was 50−1000 μg (r^2 = 0.9997). **Figure 4** shows HPLC chromatograms of TA (200 μg) before (**Figure 4A**) and after (**Figure 4B**) tannase treatment (hydrolyzed with 50 units of tannase at 30 °C for 2 h). As shown in **Figure 4A**, tannic acid has several peaks (retention time =

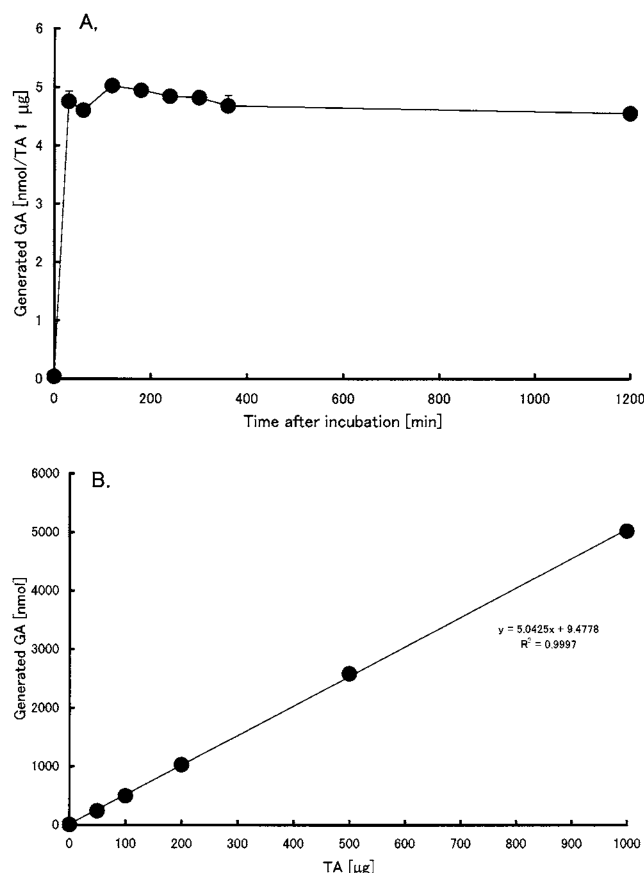

Figure 3. Hydrolysis of TA to GA by tannase: (A) tannase-mediated generation of GA from TA as a function of time; (B) calibration curve of TA (hydrolyzed with 50 units of tannase at 30 °C for 2 h). Values are means, and the vertical lines indicate SD for three trials.

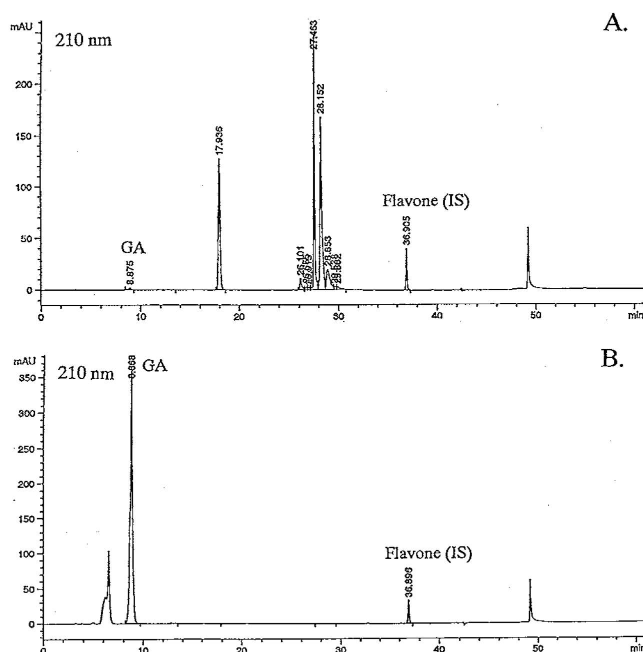

Figure 4. HPLC chromatograms of TA before (A) and after (B) tannase treatment. 200 μg of TA was treated with 50 units of tannase at 30 °C for 2 h. Produced GA (TA hydrolysate) was extracted with ethyl acetate after the incubation mixture was acidified with HCl and determined by HPLC. Conditions for HPLC are indicated in **Table 1**.

17.9 and 26.1−29.9 min), of which the UV spectra are almost the same as that of GA (data not shown). These peaks are presumably various forms of gallotannin and appear to be completely hydrolyzed to GA after tannase treatment (**Figure 4B**).

Tannase reactivity toward various polyphenols is shown in **Table 2**. Tannase hydrolyzed gallotannins such as TGG, PGG, and TA to generate GA. Tannase also hydrolyzed CG, ECG, GCG, and EGCG to produce GA and CT, GA and EC, GA and GC, and GA and EGC, respectively. Tannase hydrolyzed green tea polyphenol to produce GA, GC, EGC, CT, and EC. On the other hand, tannase was not active toward GC, EGC, or EA. The different sources of TA were also compared. All of the TA, that is, TA used in the experiments and food additives 1 and 2, were hydrolyzed completely to produce GA (HPLC chromatograms are not shown). The content of produced GA (w/w %) varied from 79.95 to 85.42%.

The recovery of TA metabolites and their related compounds from serum, urine, and feces is shown in **Table 3**. The recovery of PH from urine and dried feces and the recovery of 3,4-OMGA

Table 2. Tannase Reactivity toward Various Polyphenols

compound[a]		MW	contents		tannase reactivity[b]	GA yield (w/w %)	detected compounds
			μg	nmol			
CG	flavan-3-ol	442.4	191.2	432.2	○	32.59 ± 0.28[c]	GA + CT
ECG	flavan-3-ol	442.4	205.6	464.7	○	31.72 ± 0.45	GA + EC
GC	flavan-3-ol	306.3	179.6	586.4	×		GC
EGC	flavan-3-ol	306.3	195.6	638.6	×		EGC
GCG	flavan-3-ol	458.4	191.6	418.0	○	32.04 ± 0.51	GA + GC
EGCG	flavan-3-ol	458.4	185.6	404.9	○	32.31 ± 0.50	GA + EGC
EA		338.1	135.5	400.8	×		
TGG	gallotannin	788.6	186.4	236.4	○	61.93 ± 1.44	GA
PGG	gallotannin	940.7	234.4	249.2	○	61.47 ± 1.36	GA
TA	gallotannin		1000		○	85.42 ± 1.38	GA
food additive TA 1	gallotannin		1000		○	82.68 ± 3.62	GA
food additive TA 2	gallotannin		1000		○	79.95 ± 2.21	GA
polyphenon-100	green tea polyphenol		204.4		○	23.58 ± 1.28	GA + GC + EGC + CT + EC

[a] Abbreviations: CG, (−)-catechin gallate; ECG, (−)-epicatechin gallate; GC, (−)-gallocatechin; EGC, (−)-epigallocatechin; GCG, (−)-gallocatechin gallate; EGCG, (−)-epigallocatechin gallate; EA, ellagic acid; TGG, 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose; PGG, 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose; TA, tannic acid; GA, gallic acid; CT, (+)-catechin; EC, (−)-epicatechin. [b] To the test tube containing each compound, 5 mL of 0.05 M citrate buffer (pH 5.5) and tannase (50 units) were added, and the mixture was incubated at 30 °C for 2 h. Tannase was inactivated by the addition of 0.5 mL of 1.0 N HCl. The hydrolysate was extracted three times with 5 mL of ethyl acetate after the addition of flavone (23.62 nmol) as an internal standard. After evaporation to dryness, the residue was dissolved in 2 mL of methanol and the hydrolysate was determined by HPLC. [c] Data are means ± SD for five trials.

Table 3. Recoveries of Tannic Acid (TA), TA Metabolites and Their Related Compounds from Serum, Urine, and Feces

compound[b]	added amount	recoveries[a] (%): serum, 0.5 mL	urine, 2 mL/50 mL for 1 day	dried feces, 0.2 g: methanol-soluble fraction	dried feces, 0.2 g: total
GA	53.2 nmol	108.3 ± 3.4	85.3 ± 2.4	94.5 ± 1.9	not determined
3-OMGA	22.1 nmol	not determined	86.2 ± 7.5	95.2 ± 1.8	not determined
4-OMGA	21.5 nmol	96.8 ± 4.3	83.1 ± 2.7	95.9 ± 1.6	not determined
3,4-OMGA	20.6 nmol	97.4 ± 3.3	66.8 ± 7.4	99.2 ± 2.3	not determined
PY	32.4 nmol	94.7 ± 3.3	87.5 ± 3.6	95.6 ± 1.6	not determined
RE	54.0 nmol	68.2 ± 3.1	87.1 ± 3.8	87.3 ± 1.5	not determined
PH	43.4 nmol	105.3 ± 2.1	60.5 ± 11.6	21.2 ± 4.9	not determined
EA	8.01 nmol	92.3 ± 9.2	97.4 ± 5.0	87.3 ± 3.4	not determined
TA[c]	100 μg	not determined	not determined	97.6 ± 3.7	
	1000 μg				81.9 ± 5.0

[a] Data are means ± SD for three to five trials. Recovery tests of TA and TA metabolites in serum, urine, and feces were performed using serum, urine, and dried feces of the control rat. For the recovery tests of TA metabolites and their related compounds, adequate amounts of GA, 3-OMGA, 4-OMGA 3,4-OMGA, PY, RE, PH, and EA were added to 0.5 mL of serum, 2 mL of urine, or 2 mL of MeOH-soluble fraction of 0.2 g of dried feces. TA metabolites were analyzed without enzymatic hydrolysis according to the method described in the text. For the recovery test of TA, 100 or 1000 μg of TA was added to 2 mL of the MeOH-soluble fraction and 0.2 g of dried feces, respectively. TA was hydrolyzed by tannase for 2 h (MeOH-soluble fraction) or 20 h (total). The generated GA was analyzed as described in the text. Recovery of GA was calculated using the calibration curve prepared from the standard TA treated in the same way. [b] Abbreviations: TA, tannic acid. Other abbreviations and conditions for HPLC are indicated in **Table 1**. [c] TA was determined after enzymatic hydrolysis by tannase.

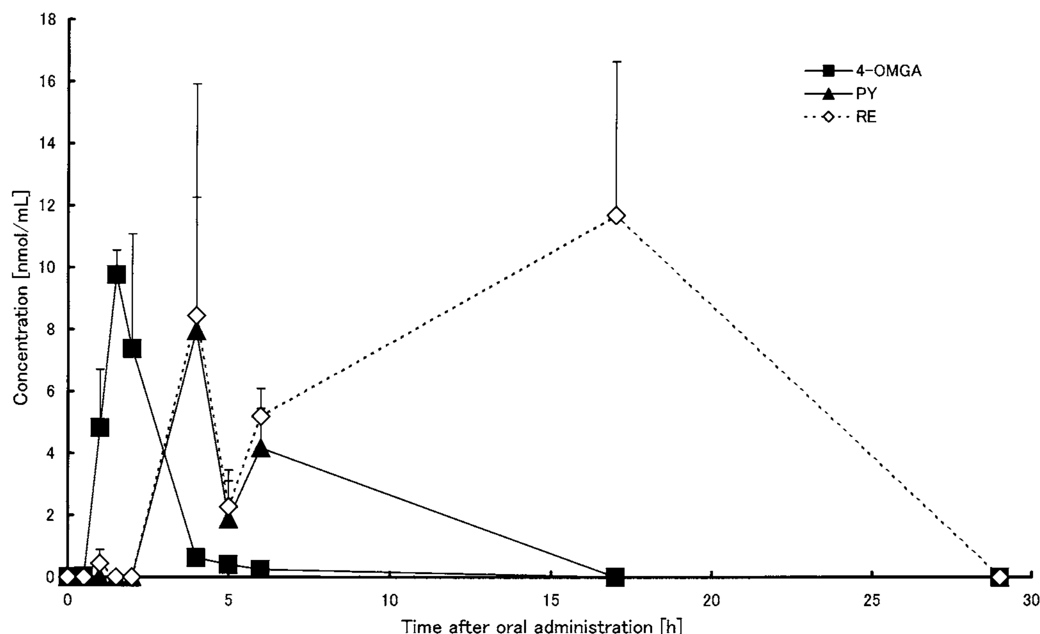

Figure 5. Serum concentrations of TA metabolites as a function of time. Serum TA metabolites were analyzed according to the method described in the text. Abbreviations: 4-OMGA, 4-*O*-methylgallic acid; PY, pyrogallol; RE, resorcinol. Values are means, and each vertical line indicates SEM for three to six rats.

from urine were low(21.2−66.8%). Nevertheless, for the other compounds, recoveries from serum, urine, and dried feces were 92.3−108.3, 83.1−97.4, and 87.3−99.2%, respectively. Recovery of TA from feces is also shown in **Table 3**. Recovery of TA was 97.6% from the MeOH-soluble fraction of feces and 81.9% from total feces. As a whole, recoveries of TA and its metabolites from serum, urine, and feces were good (>81.9%). Therefore, we analyzed TA and TA metabolites in serum, urine, and feces by using the proposed method.

Metabolism of TA in the Rat. Figure 5 shows the concentrations of TA metabolites in serum as a function of time. Only 4-OMGA, PY, and RE were detected in serum. Concentrations of 4-OMGA, PY, and RE reached maximal values at 1.5, 4, and 17 h after oral administration, respectively. These metabolites disappeared from serum 29 h after the administration of TA.

Figure 6 shows urinary concentrations of TA metabolites with or without hydrolysis by β-glucuronidase and sulfatase as a function of time. GA, 4-OMGA, PY, and RE were detected in urine. Most of the TA metabolites were excreted within 24.5 h after TA administration: GA, 100%; 4-OMGA, 95.7%; PY, 100%; and RE, 92.6%. The percentages of the free forms of urinary TA metabolites excreted within 54 h of TA administration were 44.4% for GA, 63.7% for 4-OMGA, 12.9% for PY, and 29.6% for RE.

Figure 7 shows fecal concentrations of TA metabolites with or without hydrolysis by β-glucuronidase and sulfatase as a function of time. GA, PY, and RE were detected as TA metabolites in feces. Most of the TA metabolites were excreted within 24.5 h after TA administration: GA, 73.5%; PY, 100%; and RE, 85.2%. The percentages of the free forms of urinary

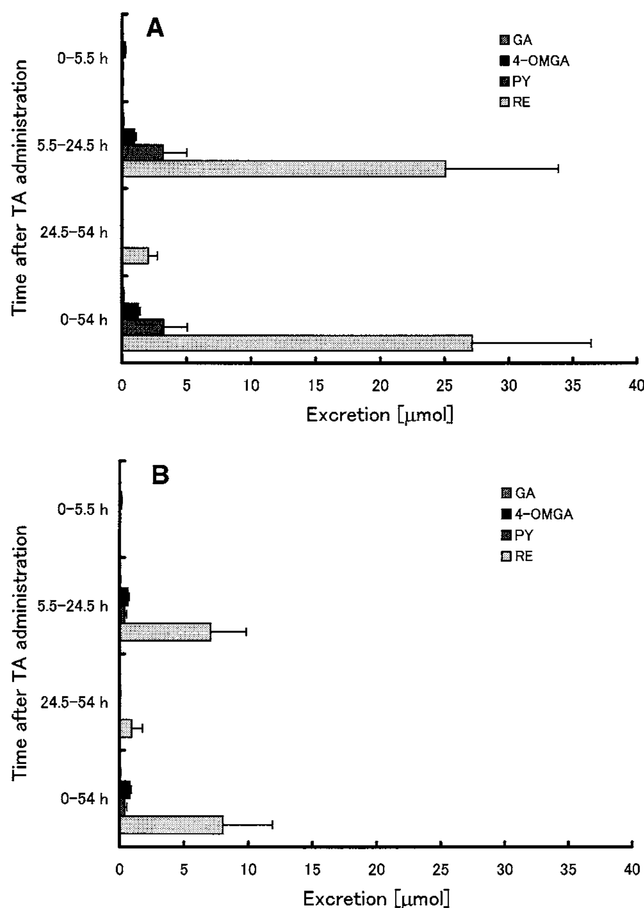

Figure 6. Urinary excretions of TA metabolites as a function of time with (A) and without (B) hydrolysis. Urinary TA metabolites were analyzed according to the method described in the text. Abbreviations: GA, gallic acid; 4-OMGA, 4-*O*-methylgallic acid; PY, pyrogallol; RE, resorcinol. Values are means, and each horizontal line indicates SEM for six rats.

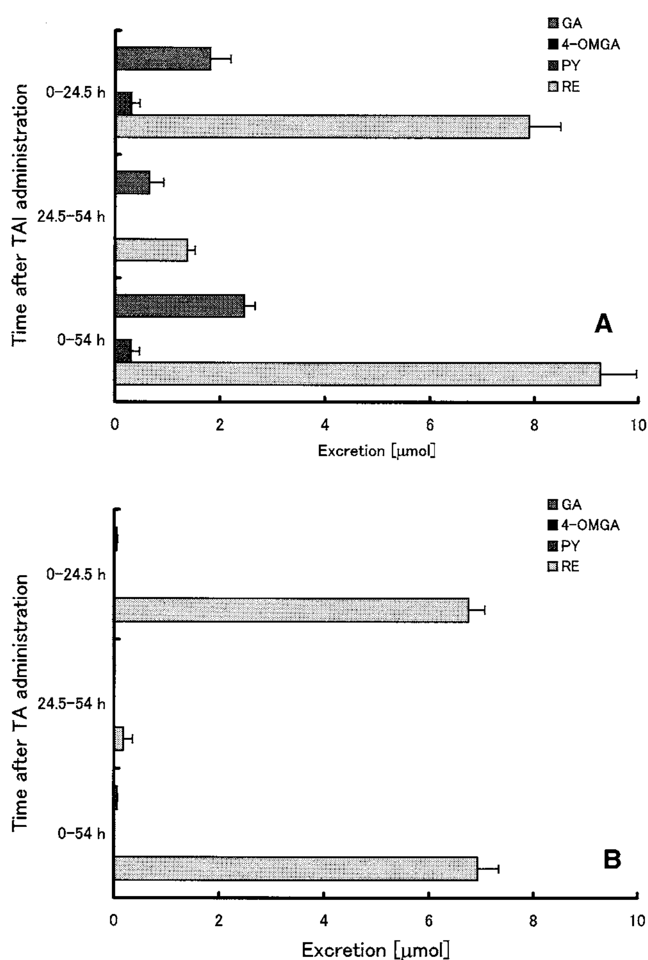

Figure 7. Fecal excretions of TA metabolites as a function of time with (A) and without (B) hydrolysis. Fecal TA metabolites were analyzed according to the method described in the text. Abbreviations: GA, gallic acid; 4-OMGA, 4-*O*-methylgallic acid; PY, pyrogallol; RE, resorcinol. Values are means, and each horizontal line indicates SEM for six rats.

TA metabolites collected 54 h after TA administration were 2.7% for GA, 0% for PY, and 74.8% for RE.

Figure 8 shows fecal excretions of TA in the total and in the MeOH-soluble fraction. TA hydrolysis by tannase was complete in 2 h for a standard solution but was incomplete for fecal TA even after a 20 h incubation as shown in **Figure 9**. Therefore, to complete hydrolysis of fecal TA to GA, the tannase treatment was done in two steps. TA in the MeOH-soluble fraction was only 0.2% of the total TA excreted, and >78.8% of total TA was excreted within 24.5 h after TA administration. The excretion rate (percent of total) of TA and its metabolites in urine and feces is shown in **Table 4**. The administered TA was calculated as GA by assuming that 1 μg of TA was equivalent to 5.022 nmol of GA based on a 2-h incubation with tannase. TA was excreted in urine as GA (0.01%), 4-OMGA (0.10%), PY (0.24%), and RE (2.06%) and in feces as TA (62.74%), GA (0.19%), PY (0.02%), and RE (0.76%) within 54 h after oral administration.

DISCUSSION

There are many kinds of dietary polyphenols for which quantitation is dependent on analytical methods (*2*, *23*, *24*). Scalbert and Williamson (*25*) reported daily intake of polyphenols as ~1 g. It is presumed that humans generally ingest polyphenols in the form of flavonoids, catechins, and proanthocyanidins, with a minor amount being gallotannins, including TA.

Before investigating the metabolism of TA in the rat, we studied an analytical method for measuring TA and TA metabolites in biological samples. Because TA metabolites are low molecular weight compounds, they were analyzed according to the method of Zhu et al. (*22*) with some modifications in chromatography. The pore size of ordinary reversed-phase HPLC columns is ~100 Å. It is possible to use these columns to determine low molecular weight compounds (<1000), but it is likely that higher molecular weight compounds are not eluted from the column. TA includes higher molecular weight compounds (1000−3000). Therefore, despite the lower sensitivity (**Table 1**), the Develosil 300 ODS-HG-5 column that has a 300 Å pore size was used to determine fecal TA and its metabolites.

The Folin−Ciocalteu method (*26*) is generally used to determine TA, but the method is not specific. TA is prone to bind proteins and forms insoluble substances (*6*). Nemoto et al. (*27*) isolated a Gram-negative tannin−protein complex-degrading bacterium from animals with the exception of the koala and showed that these bacteria produced tannase. Rajakumar and Nandy (*28*) reported that TA and chebulinic acid were first hydrolyzed by tannase liberated by *Penicillium chrysogenum* to an intermediate product, the GA ester of glucose, which was subsequently converted into GA. They also showed that an oxidase produced by the fungi decomposed the GA formed into oxalic acid (*28*). Because we were unaware of any reports on the analysis of TA that binds to biological

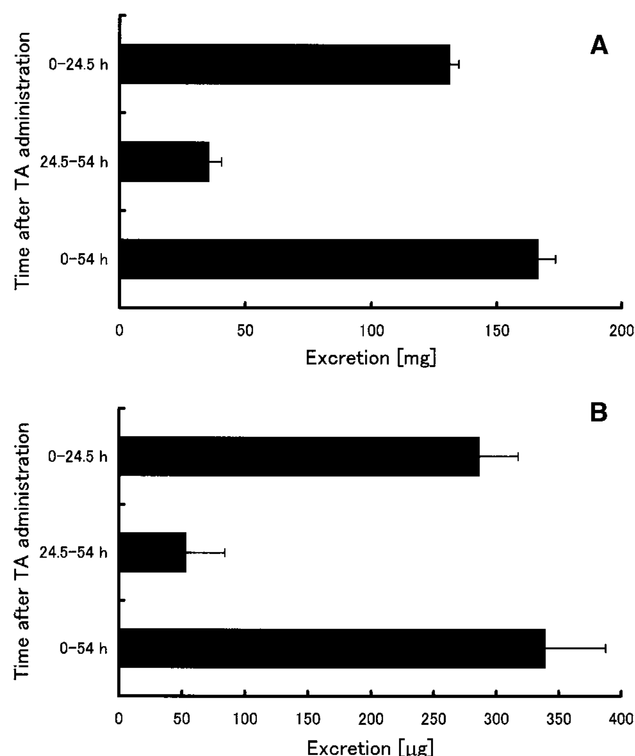

Figure 8. Fecal excretions of TA as a function of time. Fecal TA was determined as produced GA by one-step (B; TA in the MeOH-soluble fraction) or two-step (A; total TA) hydrolysis with tannase as described in the text. Values are means, and each horizontal line indicates SEM for six rats.

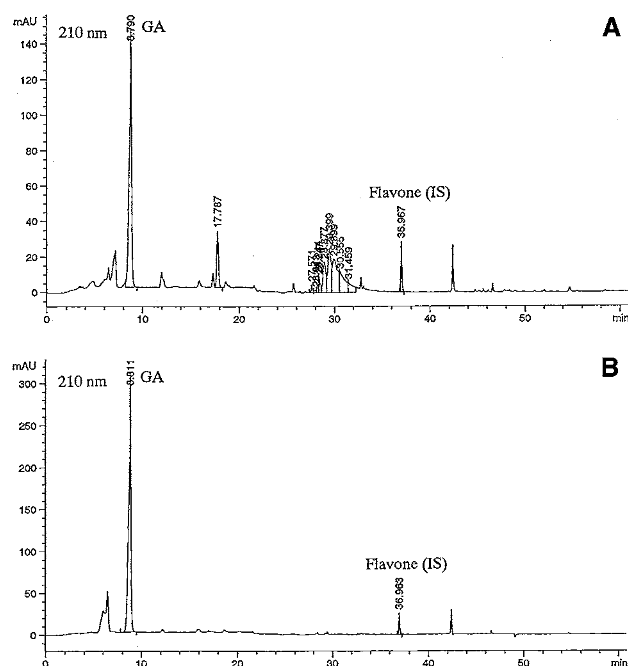

Figure 9. HPLC chromatogram of fecal total TA. Fecal total TA was analyzed by the method described in the text. (A) one-step (incomplete) hydrolysis (dried feces was treated with 2000 units of tannase at 30 °C for 20 h after the fecal lipid was removed with *n*-hexane) (B) two-step (complete) hydrolysis (further tannase treatment (50 U, 30 °C for 2 h) was performed after the one-step hydrolysis).

substances, we examined a new analytical method for TA using tannase and applied it to fecal TA analysis.

Tannase hydrolyzes the GA ester bond of various polyphenols such as TA, TGG, PGG, CG, ECG, GCG, EGCG, and green tea polyphenol and generates GA and other products (**Table 2**). As shown in **Figure 3**, the hydrolysis of TA by tannase was relatively rapid and stable over the course of 20 h. On the other hand, an acid hydrolysis of TA was problematic. The amount of GA produced reached a plateau after 8 h of treatment with 10 N HCl at 100 °C (data not shown). TA was not hydrolyzed to GA at all by acid hydrolysis during an 8 h treatment with 1 N HCl at 37 °C (data not shown). Therefore, it was presumed that the administered TA was hydrolyzed to GA by tannase produced by intestinal bacteria rather than hydrolyzed by gastric HCl. Tannase also hydrolyzes the GA ester of certain catechins and gallotannins other than TA (**Table 2**). Tannase likely acts on the GA esters of various polyphenols and produces GA in the intestinal tract. Good recoveries of TA metabolites, except for PH from dried feces (**Table 3**), show that the proposed method is applicable to the determination of TA and its metabolites in biological samples. To our knowledge, this is the first study that uses tannase for TA analysis.

Serum TA metabolites were analyzed only following treatments with β-glucuronidase and sulfatase because they were present in trace amounts. The time needed to reach a maximum concentration of serum TA metabolites was dependent on the particular compound: 4-OMGA was the fastest, PY was intermediate, and RE was the slowest (**Figure 5**). Four kinds of TA metabolites, that isi.e., GA, 4-OMGA, PY, and RE, were detected from urine (**Figure 6**), but 4-OMGA was not detected from feces. This suggests that 4-OMGA was produced from GA in the liver and excreted into urine and was not produced in the intestinal tract. Of all the TA metabolites detected in urine and feces, the disappearance of PY was the fastest, as it was completely excreted within 24.5 h. Although the amounts of the total TA metabolites was RE $\gg$ PY > 4-OMGA > GA in urine and RE $\gg$ GA > PY in feces, the relative abundance of the free forms of the TA metabolites, that is, not conjugated with glucuronides or sulfates, was found to be 4-OMGA > GA > RE > PY in urine and RE > GA > PY in feces (**Figures 5**

Table 4. Excretion Rate of TA and Its Metabolites in Urine and Feces

	excretion rate[a] (% of total)						
	in feces			in urine			
compound[b]	0–24.5 h	24.5–54 h	0–54 h	0–5.5 h	5.5–24.5 h	24.5–54 h	0–54 h
TA	49.48 ± 1.47	13.26 ± 1.88	62.74 ± 2.36				
GA	0.16 ± 0.02	0.03 ± 0.01	0.19 ± 0.02	0.00 ± 0.00	0.01 ± 0.00	ND[c]	0.01 ± 0.00
4-OMGA	ND	ND	ND	0.02 ± 0.00	0.07 ± 0.01	ND	0.10 ± 0.01
PY	0.02 ± 0.01	ND	0.02 ± 0.01	0.00 ± 0.00	0.24 ± 0.13	ND	0.24 ± 0.13
RE	0.65 ± 0.04	0.10 ± 0.01	0.76 ± 0.04	0.00 ± 0.00	1.87 ± 0.65	0.18 ± 0.05	2.06 ± 0.70

[a] Data are means ± SEM for six rats. [b] Abbreviations: TA, tannic acid; GA, gallic acid; 4-OMGA, 4-*O*-methylgallic acid; PY, pyrogallol; RE, resorcinol. [c] ND, not detected.

and **6**). The biological significance of glucuronization and/or sulfation in vivo is thought to be the conversion of metabolites to more hydrophilic and less toxic forms. That is, it was supposed that the more free form, the less toxic. Therefore, it might be possible that PY is the most toxic metabolite and that 4-OMGA is the least.

Of the administered TA, 0.97 and 2.41% were excreted as TA metabolites (GA, 4-OMGA, PY, and RE) in feces and urine, respectively (**Table 4**). Zong et al. (*29*) reported that GA and its metabolite 4-OMGA appeared in serum and urine in the rat. They stated that the concentration of 4-OMGA in urine reached a maximum 3 h after GA administration. Sharzad et al. (*30*) reported that 36.4% of administered GA was excreted into urine as GA and 4-OMGA in humans. The GA content of the administered TA was <0.3%. Thus, it was suggested that GA was generated de novo in the intestine from the administered TA by tannase of the intestinal microflora. It is possible that the generated GA from TA was absorbed from the intestine and further metabolized to 4-OMGA, probably in the liver and/or kidney. It is also possible that some of the generated GA was further degraded to RE by the intestinal bacteria and then absorbed, rather than being produced as a metabolite of GA in the liver. The metabolic pathway of GA to PY or RE might be different from the metabolic pathway of GA to 4-OMGA. Some fraction of the absorbed TA metabolites from the intestine might be conjugated to glucuronides and/or sulfates to reduce their toxicity.

Of the administered TA, 62.7% was excreted into feces as TA (**Table 4**). GA extracted from feces without tannase treatment was negligible compared with GA extracted from feces with tannase treatment. The reason hydrolysis of fecal TA to GA by tannase was incomplete in one step (**Figure 9**) is unknown. Because only 0.2% of total fecal TA existed in the MeOH-soluble fraction (**Figure 8**), it is possible that most of the administered TA is combined with the undigested substances or fecal microflora. The proposed method for TA analysis using tannase in this study revealed its applicability to the determination of bound TA. Bravo et al. (*7*) reported that 4.6% of administered TA was excreted into feces, but they determined only soluble polyphenols by the Folin−Ciocalteu method. Carbonaro et al. (*31*) showed that the disappearance of TA in the small intestine in rat was 50%. This is the first report to describe the metabolism of TA in the rat including a determination of overall TA binding.

In conclusion, 66.12% of administered TA was excreted as TA and its metabolites into feces and urine: 62.74% as TA and 0.97% as TA metabolites into feces and 2.41% as TA metabolites into urine, when TA was administered to the rat as a single dose at 1.0 g/kg to the rat. Nondetection of 33.78% of the administered TA might be accounted for in two ways. One might be further degradation of GA in the intestinal tract, entry into the TCA cycle, and finally expiration as CO_2; another might be the difficulty of extracting bound TA in feces. These explanations do not rule out other possibilities.

Singh et al. (*32*) showed that the microbial degradation products of TA by ruminal fluid of cattle were GA and PY after 24 h of incubation and PY and RE after 48 and 72 h of incubation. Zhu et al. (*10*) reported that GA, 4-OMGA, EA, and TA were detected in plasma and GA, PY, and 4-OMGA were detected in urine in sheep by abomasal dosing of TA. They also showed that the plasma concentrations of GA, 4-OMGA, EA, and TA and urinary concentrations of GA and 4-OMGA reached maximal values 8 h after administration and that urinary PY concentration reached maximal values 12 h after TA administration (*10*). In our study, no EA or TA was detected from serum (**Figure 5**). A rat does not have an abomasum or a rumen because the rat is an omnivore, not a ruminant. Therefore, TA metabolism might differ by species.

Although future use of polyphenols in the human diet may increase because of their known beneficial properties (*33*), their metabolism, interactions with other nutrients, and safe intake levels remain to be clarified.

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