Urinary Metabolites of Gallic Acid in Rats and Their Radical-Scavenging Effects on 1,1-Diphenyl-2-picrylhydrazyl Radical

Takaaki Yasuda, Asako Inaba, Masaki Ohmori, Tomoko Endo, Sayaka Kubo, and Keisuke Ohsawa*

Department of Phytochemistry, Tohoku Pharmaceutical University, 4-1 Komatsushima 4 Chome, Aoba-ku, Sendai, Japan 981-8558

Received January 31, 2000

As a part of our studies on the metabolism of natural compounds, gallic acid was orally administered to rats. The urinary metabolites were analyzed by high-performance liquid chromatography, and their structures were determined to be pyrogallol (**M1**), pyrogallol-1-*O*-*β*-D-glucuronide (**M2**), 4-*O*-methylgallic acid-3-*O*-sulfate (**M3**), 2-*O*-methylpyrogallol-1-*O*-*β*-D-glucuronide (**M4**), 2-*O*-methylpyrogallol (**M5**), 4-*O*-methylgallic acid (**M6**), and unchanged gallic acid on the basis of chemical and spectral data. The radical scavenging effects of gallic acid and its urinary metabolites were evaluated using 1,1-diphenyl-2-picrylhydrazyl radical.

Gallic acid is widely distributed in plants. The bound forms of gallic acid, notably epicatechin gallate or galloyl glucosides and tannic acids, are associated with and probably are the main source of free gallic acid in foods.[1] Gallic acid has been recently recognized as possessing antioxidative, antiallergic, antiinflammatory, antimutagenic, and anticarcinogenic activities.[1−6] The metabolism and pharmacokinetics of gallic acid have been investigated in various animal species.[1,7−12] Recently, Shahrzad et al.[13] investigated the metabolism of gallic acid in humans by HPLC method in which 4-*O*-methylgallic acid and unchanged gallic acid were detected in hydrolyzed urine and plasma. They showed that these metabolites were mainly present as conjugates in human urine and plasma. However, no report has appeared on the conjugated position of their metabolites. The effects of many drugs can be attributed wholly or partially to the action of their metabolites, rather than the direct action of the drug. On the other hand, in addition to the attention focused on drug/drug interactions in multiple-drug therapy, drug/metabolite(s) interactions should be noted in pharmacokinetic mass-balance studies, especially for the case in which the regeneration of a drug from the conjugated metabolites is significant. Precise information regarding the pharmacokinetics of the conjugated metabolite itself is indispensable for the kinetic investigation of such a drug/conjugated-metabolite interaction study.

In the present paper, as a part of our studies on the metabolism of natural compounds, we report the structures of urinary metabolites of orally administered gallic acid in rats. Furthermore, we examined the scavenging effects of gallic acid and its urinary metabolites on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, frequently used as a model compound for free radicals in lipids.[14]

A three-dimensional HPLC profile of the urine sample collected from the rats after oral administration of gallic acid revealed the presence of seven distinct peaks, which were named **M1**, unchanged gallic acid, **M2**, **M3**, **M4**, **M5**, and **M6**, in decreasing order of polarity (see Figure 1).

The isolation of **M1**−**M6** and unchanged gallic acid from the urine was performed by chromatographic separation on a Sephadex LH-20 column and repeated preparative HPLC as described in the Experimental Section. The structures of **M1**−**M6** and unchanged gallic acid were determined as below.

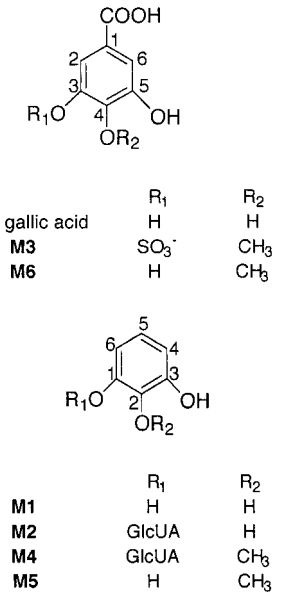

Figure 1. Urinary metabolites of gallic acid in rat.

Unchanged gallic acid was identified by direct comparison with an authentic sample. **M1** and **M6** were identified as pyrogallol and 4-*O*-methylgallic acid by direct comparison with authentic samples, respectively.

M2 was obtained as colorless oil, and its negative FABMS showed a fragment ion peak corresponding to $[M - H + Na]^-$ at *m*/*z* 323. The enzymatic hydrolysis of **M2** with *β*-glucuronidase gave pyrogallol, which was identified by direct comparison with an authentic sample (EIMS and ^{1}H NMR data). The ^{1}H NMR spectrum showed one anomeric proton at *δ* 4.65, supporting the presence of one glucuronide group in **M2**. The position of attachment of the glucuronic acid moiety was confirmed by the HMBC experiment, in which long-range correlations were observed between the anomeric proton of glucuronic acid at *δ* 4.65 and C-1 at *δ* 146.1, indicating the glucuronide group is attached at C-1. Based on these data, **M2** was determined to be pyrogallol-1-*O*-*β*-D-glucuronide.

The negative-ion FABMS of **M3** showed an ion peak corresponding to $[M - H]^-$ at *m*/*z* 263 along with fragment

* To whom correspondence should be addressed. Tel.: 022-234-4181. Fax: 022-275-2013. E-mail: ohsawa@tohoku-pharm.ac.jp.

10.1021/np0000421 CCC: $19.00
Published on Web 08/30/2000

ion peaks at *m/z* 285 $[M - H + Na]^-$ and 183 $[M - H - SO_3]^-$. Enzymatic hydrolysis of **M3** with arylsulfatase gave **M6**, indicating the presence of a sulfate group in **M3**. A comparison of the ^{13}C NMR spectrum of **M3** with that of **M6** indicated the C-3 signal of **M3** to have shifted 3.1 ppm upfield, accompanied by downfield shifts of C-2 (5.6 ppm) and C-4 (5.8 ppm), indicating the sulfate group to be situated at C-3. The structure of **M3** was concluded to be 4-*O*-methylgallic acid-3-*O*-sulfate.

M4 was obtained as a colorless oil, and its negative FABMS showed a fragment ion peak corresponding to $[M - H + Na]^-$ and $[M - H]^-$ at *m/z* 337 and 315. The enzymatic hydrolysis of **M4** with β-glucuronidase gave 2-*O*-methyl-pyrogallol (**M5**), which was identified by reference data[15] (MS, ^1H and ^{13}C NMR data). The ^1H NMR spectrum showed one anomeric proton at δ 4.79, and the position of attachment of the glucuronic acid moiety as C-1 was confirmed by the HMBC experiment, in which long-range correlations were observed between the anomeric proton of glucuronic acid at δ 4.79 and C-1 at δ 150.8. Based on these data, **M4** was determined to be 2-*O*-methylpyrogallol-1-*O*-β-D-glucuronide. **M5** was identified as 2-*O*-methylpyrogallol by comparison with the reference data[15] (MS, ^1H and ^{13}C NMR data).

The metabolism and pharmacokinetics of gallic acid have been reported in various animal species. In rats, rabbits, and chickens, the major urinary metabolite is 4-*O*-methylgallic acid and the secondary product is pyrogallol (conjugated and unconjugated).[1,7−11] Small amounts of conjugated 2-*O*-methylpyrogallol as a third metabolite of gallic acid were also detected in rats.[7,9] In sheep, resorcinol glucuronide is the major product of gallic acid metabolism, and the minor urinary metabolites are unconjugated pyrogallol and resorcinol.[12] Recently, Shahrzad et al.[13] investigated the metabolism of gallic acid in humans using HPLC and, after hydrolysis, 4-*O*-methylgallic acid and unchanged gallic acid were detected in urine and plasma, illustrating that these metabolites exist mainly as conjugates in human urine and plasma. However, no report has appeared on the position and manner of conjugation of the metabolites. We report herein the structures of the conjugated forms of rat urinary metabolites.

The radical scavenging activities of gallic acid and its urinary metabolites, together with the well-known antioxidants DL-α-tocopherol and ascorbic acid, were investigated. Gallic acid ($EC_{50} = 4.2\ \mu M$) and **M1** ($EC_{50} = 4.2\ \mu M$) showed the most potent scavenging effect, with greater activity than either DL-α-tocopherol ($EC_{50} = 19.9\ \mu M$) or ascorbic acid ($EC_{50} = 16.6\ \mu M$). Gallic acid and **M1** showed the same radical scavenging activity, suggesting that the carboxylic acid in the gallic acid molecule has no effect on radical scavenging. Furthermore, the observed values correlate well with a previous observation[14] that gallic acid scavenged the superoxide anion radical at lower concentration than DL-α-tocopherol. On the other hand, this result also indicates that the radical scavenging effects of the conjugated form [**M2** ($EC_{50} = 52.0\ \mu M$), **M3** ($EC_{50} = >100\ \mu M$), and **M4** ($EC_{50} = >100\ \mu M$)] and/or the methylated derivatives [**M5** ($EC_{50} = 99.6\ \mu M$) and **M6** ($EC_{50} = >100\ \mu M$)] are weaker than each free form. From these results, it was shown that the number of phenolic free hydroxyl groups seems to be important for radical scavenging activity.

Experimental Section

Apparatus. Melting points were determined on Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were measured with a Perkin-Elmer FT-IR 1725X spectrometer. NMR spectra were recorded on a JEOL JNM-EX 270 with tetramethylsilane as an internal standard, and the chemical shifts are given as δ values. Mass spectra were measured with a JEOL DX-303 mass spectrometer. HPLC system consisted of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan), and Waters model 991J photodiode array detector (Waters Millipore, Milford, MA).

Reagents. Gallic acid and pyrogallol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). DPPH, DL-α-tocopherol, and ascorbic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). β-Glucuronidase and arylsulfatase were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals. Male SD rats (Japan SLC Inc.), 6 weeks old, were used. These animals were maintained under a constant temperature (22 ± 2 °C) and humidity (55 ± 10%), with free access to commercial diet (CA-1, Clea Japan, Tokyo) and tap water in a room with a 12-h light/12-h dark cycle. They were deprived of food but had free access to water for 18 h prior to the experiments.

Preparation of Urine Samples. Gallic acid (200 mg/kg) dissolved in 0.5% sodium carboxymethylcellulose solution were administered orally to 60 rats, and urine specimens were obtained over 24 h by using a metabolic cage. Methanol (5 mL) was added to 1 mL of the urine, and the mixture was filtered through a 0.45-μm filter, and then 20 mL of the sample was injected into the HPLC column.

HPLC Conditions. A stainless steel column (0.46 × 25.0 cm i.d.), packed with reversed-phase TSKgel ODS-120T (5 μm, Tosoh Company Ltd., Tokyo, Japan) were used. The mobile phase was the following linear gradient system: solvent A, 0.05% trifluoroacetic acid (TFA); solvent B, 100% acetonitrile with 0.05% TFA, A/B = 100/0 (0 min) → 90/10 (50 min) → 90/10 (60 min). Flow rate 1.0 mL/min at 40 °C. The t_R values for **M1**−**M6** were 11.2, 12.9, 20.9, 24.1, 27.8, and 31.3 min, respectively.

Isolation of Urinary Metabolites. For the isolation of urinary metabolites, 2.8 g of gallic acid was orally administered at 200 mg/kg to 60 rats, and urine samples were collected by using metabolic cages. The combined urine sample (510 mL) collected from gallic acid-treated rats was chromatographed on Sephadex LH-20 (0.05 N HCl → H_2O → MeOH), and the fractions containing metabolites were subjected to preparative HPLC. The conditions were as follows: column, TSKgel ODS-120T (10 μm, 0.78 × 30.0 cm i.d., Tosoh Company Ltd., Tokyo, Japan); mobile phase, solvent A, 20% methanol; solvent B, 100% methanol; linear gradient system, A/B = 100/0 (0 min) → 40/60 (100 min); flow rate, 2.0 mL/min at room temprature. Each metabolite fraction was purified by Sephadex LH-20 column chromatography (H_2O) and to give **M1** (30 mg), **M2** (17 mg), **M3** (8 mg), **M4** (15 mg), **M5** (19 mg), **M6** (20 mg), and gallic acid (6 mg).

Enzymatic Hydrolysis of Metabolites. M2 (10 mg) and **M4** (6 mg) were incubated with β-glucuronidase (type H-2) in 0.1M citrate buffer (pH 5.2) for 2 h at 37 °C. **M3** (5 mg) was treated with arylsulfatase (type H-1) in the same way as above. Each reaction mixture was purified by Sephadex LH-20 column chromatography to give pyrogallol (4 mg), 2-*O*-methylpyrogallol (3 mg), and 4-*O*-methylgallic acid (2 mg), respectively.

Radical Scavenging Effect on DPPH Radical. A MeOH solution of test compound was added to a solution of DPPH (1.5×10^{-14} M) in MeOH (1 mL), and the reaction mixture was shaken vigorously. After the solution was left to stand for 30 min, the absorbance of the resulting solution at 520 nm was measured. The scavenging activity on the DPPH radical was expressed as EC_{50}, the concentration of the test compound required to give a 50% reduction in the absorbance from that of 1.5×10^{-14} M DPPH in MeOH.

M2 (pyrogallol-1-*O*-β-D-glucuronide): colorless oil, IR (NaCl) ν_{max} 3425, 1684 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz) δ 4.65 (1H, d, J = 6.8 Hz, anom-H), 6.49 (1H, dd, J = 8.0, 2.0 Hz, H-4), 6.54 (1H, t, J = 8.0 Hz, H-5), 6.58 (1H, dd, J = 8.0,

2.0 Hz, H-6); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 71.7 (GlcUA C-4), 73.0 (GlcUA C-2), 74.3 (GlcUA C-5), 75.5 (GlcUA C-3), 103.0 (GlcUA C-1), 108.8 (C-6), 110.8 (C-4), 118.2 (C-5), 135.5 (C-2), 146.1 (C-1), 146.2 (C-3), 171.9 (GlcUA C-6); FABMS *m*/*z* 323 [M − H + Na]$^-$ (15), 155 (100).

M3 (4-*O*-methylgallic acid-3-*O*-sulfate): white powder, mp 186−187 °C. IR (KBr) ν_{max} 3470, 1510 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 3.78 (3H, s, OCH_3), 7.14 (1H, d, J = 2.1 Hz, H-6), 7.60 (1H, d, J = 2.1 Hz, H-2); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 60.0 (O*C*H$_3$), 111.9 (C-6), 114.1 (C-2), 125.2 (C-1), 142.6 (C-3), 146.4 (C-4), 150.0 (C-5), 167.1 (*C*OOH); FABMS *m*/*z* 285 [M − H + Na]$^-$ (100), 263 (96), 183 (30); HRFABMS *m*/*z* 263.9990 (calcd for $C_8H_8O_8S$, 263.9940).

M4 (2-*O*-methylpyrogallol-1-*O*-*β*-D-glucuronide): colorless oil, IR (NaCl) ν_{max} 3384, 1685 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 3.72 (3H, s, OCH_3), 4.79 (1H, d, J = 6.8 Hz, anom-H), 6.51 (1H, dd, J = 8.2, 1.5 Hz, H-6), 6.59 (1H, dd, J = 8.2, 1.5 Hz, H-4), 6.79 (1H, t, J = 8.2 Hz, H-5); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 60.1 (O*C*H$_3$), 71.9 (GlcUA C-4), 73.1 (GlcUA C-2), 73.6 (GlcUA C-5), 76.7 (GlcUA C-3), 101.1 (GlcUA C-1), 107.6 (C-4), 110.2 (C-6), 123.2 (C-5), 137.3 (C-2), 150.8 (C-1), 151.5 (C-3), 172.4 (GlcUA C-6); FABMS *m*/*z* 337 [M − H + Na]$^-$ (15), 315 (8), 205 (100).

References and Notes

(1) Singleton, V. L. *Adv. Food Res.* **1981**, *27*, 149−172.
(2) Gali, H. U.; Perchellet, E. M.; Klish, D. S.; Johnson, J. M.; Perchellet, J. P. *Carcinogenesis* **1992**, *13*, 715−718.
(3) Hayatsu, H.; Arimoto, S.; Negishi, T. *Mutat. Res.* **1988**, *202*, 429−446.
(4) Stich, H. F.; Rosin, M. P. *Adv. Exp. Med. Biol.* **1984**, *177*, 1−29.
(5) Perchellet, J. P.; Gali, H. U.; Perchellet, E. M.; Klish, D. S.; Armbrust, A. D. *Basic Life Sci.* **1992**, *59*, 783−801.
(6) Gali, H. U.; Perchellet, E. M.; Perchellet, J. P. *Cancer Res.* **1991**, *51*, 2820−2825.
(7) Scheline, R. R. *Acta Pharmacol. Toxicol.* **1966**, *24*, 275−285.
(8) Potter, D. K.; Fuller, H. L. *J. Nutr.* **1968**, *96*, 187−191.
(9) Booth, A. N.; Masri, M. S.; Robbins, D. J.; Emerson, O. H.; Jones, F. T.; Deeds, F. *J. Biol. Chem.* **1959**, *234*, 3014−3016.
(10) Watanabe, A.; Oshima, Y. *Agric. Biol. Chem.* **1965**, *29*, 90−93.
(11) Glick, Z. *J. Nutr.* **1981**, *111*, 1910−1916.
(12) Murdiati, T. B.; McSweeney, C. S.; Lowry, J. B. *Aust. J. Agric. Res.* **1992**, *43*, 1307−1319.
(13) Shahrzad, S.; Bitsh, I. *J. Chromatogr. B.* **1998**, *705*, 87−95.
(14) Hatano, T.; Edamatsu, R.; Hiramatsu, M.; Mori, A.; Fujita, Y.; Yasuhara, T.; Yoshida, T.; Okuda, T. *Chem. Pharm. Bull.* **1989**, *37*, 2016−2021.
(15) Green, K. *J. Org. Chem.* **1991**, *56*, 4325−4326.

NP0000421