Metabolic Fate of Gallic Acid Orally Administered to Rats

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The metabolic behavior of orally administered gallic acid was investigated by HPLC and 4-O-methyl gallic acid was found to be the major metabolite in rat peripheral blood and urine. After oral administration of gallic acid, maximum concentration in portal vein and inferior vena cava occurred at 15 and 30 min, respectively. In portal vein, gallic acid was preferentially detected relative to 4-O-methyl gallic acid, whereas gallic acid and 4-O-methyl gallic acid were equally detected in inferior vena cava. On the other hand, 4-O-methyl gallic acid but not gallic acid was found in liver. The contents of gallic acid and 4-O-methyl gallic acid in urine were nearly 100 times higher than those in blood. The ratio of 4-O-methyl gallic acid to total gallic acid metabolites in urine was from 0.55 to 0.76, indicating that a considerable amount of gallic acid was excreted without being metabolized. In this study we found that gallic acid administered orally existed in the blood for 6 h at most, and more than half was metabolized to 4-O-methyl gallic acid, followed by excretion into urine.

Key words gallic acid; 4-O-methyl gallic acid; metabolism; pyrogallol

Gallic acid (3,4,5-trihydroxybenzoic acid) occurs in many medicinal plants in free form or in a conjugated form, such as found in hydrolysable tannins. Gallic acid is known to show many pharmacological and biochemical activities: for example, antiinflammatory activity against zymosan-induced acute food pad swelling in mice, inhibition of adenoma induction by morphine and NaNO₃, and growth inhibitory activity against HeLa and Raji cells. In our laboratory, we isolated gallic acid as an anti-cancer agent from chestnut bark (Juglans mandshurica Maxim.), which is used as an ingredient of Chinese herbal medicine for therapy of hepatic cancer and chronic cirrhosis. In addition, we determined that gallic acid induces apoptosis in various cancer cells at concentrations of around 6 μg/ml, and that the effect resulting in cell death of cancer cells is more potent than that against normal cells. We have considered whether gallic acid is actually useful as an anti-cancer agent that can induce apoptosis in cancer cells in vivo. In attempting application of gallic acid as a therapeutic agent against cancer, a study of metabolism in the body is required in order to choose the best method of administration. However, such a study has so far been limited to the analysis of urine after gallic acid administration. For instance, Booth showed that 4-O-methyl gallic acid and pyrogallol were identified as the metabolites of gallic acid in urine after intraperitoneal injection of gallic acid into rats. Scheline also reported that pyrogallol was found in urine after gallic acid was administered orally. In addition, there are several studies concerning the metabolism of related substances such as octyl gallate, propyl gallate and tannic acid, which revealed that gallic acid was detected in urine when these compounds were given orally to rats.

Therefore, in this study we attempted to investigate the metabolic fate of gallic acid in peripheral blood, liver and urine after oral administration of gallic acid in order to determine the most proper route of administration to treat liver cancer.

MATERIALS AND METHODS

Reagents Gallic acid was obtained from Nakalai Tesque Co. and recrystallized from water for use in this study. 4-O-Methyl gallic acid was synthesized and refined according to the method of Wymann. Pyrogallol was provided by Nakalai Tesque Co.

High Performance Liquid Chromatography (HPLC) A Shimadzu Model 6A chromatograph with a Shimadzu Model SPD-M6A photodiode array UV-VIS detector was used, and a Lichrocart RP-18 column (5 μm, i.d. 4.0 × 250 mm, Merck Co., Germany) for analysis and Lichrosorb RP-18 column (5 μm, i.d. 10 × 250 mm, Merck Co., Germany) for preparative HPLC were used. The mobile phase employed for analysis was a gradient of CH₃CN/1.5 mM H₃PO₄ at 1.0 ml/min as follows: initial conditions 2% CH₃CN for 10 min, followed by sequential gradients to 21% CH₃CN for 20 min. For preparative HPLC, compounds were eluted with a gradient of CH₃CN/1.5 mM H₃PO₄ at 1.0 ml/min as follows: initial conditions 10% CH₃CN for 8 min, followed by sequential gradients to 60% CH₃CN for 37 min. The column temperature was 30°C and the detection wavelength was 260 nm.

Animals 6 week old male Wistar rats were purchased from Shizuoka laboratory animal Center (Hamamatsu, Japan). They were fed a laboratory chow (CE-2, Clea Japan Inc., Tokyo, Japan) and tap water ad libitum and were housed in a temperature-controlled room (23±1°C) with lighting from 7 a.m. to 7 p.m.

Determination of Metabolites Gallic acid was orally administered to rats at concentrations of 50, 100, and 500 mg/kg. Blood samples were taken from portal vein and inferior vena cava at 15, 30, 60, and 180 min after administration, while rats were under light anesthesia with ether. To avoid the mixing of blood from portal vein and inferior vena cava, 2 ml of blood was first drawn from inferior vena cava and then 1 ml of blood was drawn from portal vein following ligatation of portal vein at a site near the liver. Sera were prepared

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by centrifugation at 3200 rpm for 15 min and stored at 
-20 °C for further analysis. Urine was collected at the 
indicated time and stored at -20 °C. Intestinal contents of rats 
were collected as follows: in order to avoid contamination 
due to enterohepatic circulation, bile duct was first ligated 
and then gallic acid was orally administered at a concentra-
tion of 100 mg/kg. Rats were killed by decapitation and 
whole intestinal contents were carefully taken from intestine 
and stored at -20 °C. Liver was removed after perfusion 
with 30 ml saline to eliminate blood contamination.

**Treatment of Samples for HPLC Analysis** For analysis 
of serum, 100 μl of serum was mixed with 50 μl of 2 N HCl 
and then 4 ml of CH₃CN was added. After vigorous mixing, 
the sample was centrifuged at 1200 rpm for 5 min and the 
resulting supernatant was filtered through a HPLC PEFT filter 
(0.45 μm). The precipitate was washed with 2 ml of CH₃CN, 
centrifuged and combined with the previous supernatant. The 
supernatant was evaporated to dryness under nitrogen gas 
and the residue was reconstituted with 200 μl of HPLC 
methanol. A 15 μl aliquot was applied to HPLC. For analysis 
of urine, 100 μl of urine was mixed with 100 μl of 2 N HCl 
and then 8 ml of CH₃CN was added. The extraction procedure 
was the same as for serum. Finally, the resulting residue 
was reconstituted with 4 ml of methanol and 10 μl was sub-
jected to HPLC.

For analysis of liver, 0.1 g of liver was exactly weighed 
and homogenized in 100 μl of 2 N HCl. 1 ml of CH₃CN was 
then added and the mixture again homogenized. After cen-
trifugation at 1200 rpm for 5 min, the supernatant was filtered 
by PEFT filter and 15 μl of the resulting supernatant was 
applied to HPLC. For determination of conversion of gallic acid 
into 4-O-methyl gallic acid by intestinal contents, 0.1 g 
of intestinal contents were added to 100 μl of 2 N HCl 
and mixed for 1 min. Thereafter, 8 ml of CH₃CN was added 
and mixed again. The extraction procedure was the same as 
for serum. Finally, 1 ml of methanol was added to dissolve 
residues. The solution was filtered again and 15 μl was ap-
plied to HPLC.

**RESULTS**

Gallic acid and its metabolites were simultaneously de-
tectable in blood as early as 15 min after oral administration 
of gallic acid. Figure 1 shows a typical HPLC profile for gal-
lic acid and its metabolites in a blood sample obtained from 
portal vein. Gallic acid appeared at 10.86 min and its 
metabolite at 26.46 min. There were no other peaks corre-
sponding to metabolites of gallic acid in the HPLC chro-
natogram. The metabolite was isolated by subsequent 
preparative HPLC and identified as 4-O-methyl gallic acid 
(the structure shown in Fig. 1) by comparison of HPLC re-
tention time, ¹H-NMR, ¹³C-NMR and mass spectrometry 
spectral data with those of the synthetic standard (data not 
shown). As shown in Fig. 2, orally administered gallic acid 
(100 mg/kg) was absorbed quickly from the alimentary tract 
and reached a maximum concentration at 15 min in portal 
vein. Thereafter, it decreased to half over the next 30 min 
and almost disappeared at 6 h after administration. On the other 
hand, 4-O-methyl gallic acid, the metabolite of gallic acid, 
also reached a maximum at 15 min and then decreased 
slowly. In inferior vena cava, both gallic acid and 4-O-methyl 
gallic acid reached a maximum at 30 min and decreased 
gradually until 6 h after the administration (Fig. 3). The con-
centrations of gallic acid and 4-O-methyl gallic acid in in-
ferior vena cava were similar to each other. When comparing 
the concentrations in portal vein and inferior vena cava, the 
concentration of gallic acid in portal vein was two times
higher than in inferior vena cava, whereas the concentration of 4-O-methyl gallic acid was almost the same. In order to examine where 4-O-methyl gallic acid was produced, we prepared intestinal content, incubated with gallic acid in vitro and then analyzed the metabolites. Although data is not shown, 4-O-methyl gallic acid was not produced from gallic acid by the incubation with intestinal contents.

We next determined the accumulation of gallic acid and its metabolites in liver. 4-O-Methyl gallic acid was found in liver homogenate prepared after thorough perfusion with saline, whereas gallic acid could not be detected (Fig. 4). We next examined the excretion of gallic acid and its metabolite into urine. The main metabolite of gallic acid in urine was 4-O-methyl gallic acid and its concentration was almost 100 times higher than that in inferior vena cava (Fig. 5). On the other hand, gallic acid was also found in urine at a higher concentration than that in inferior vena cava, but at a lower concentration than that of 4-O-methyl gallic acid. We were not able to find pyrogallol in urine, in contrast with a previous report. When the ratio of the conversion of gallic acid to 4-O-methyl gallic acid was calculated using urine samples, it ranged from 55.3 to 76.5% (Table 1), which was similar to that in inferior vena cava, but was higher than that in portal vein.

### Table 1. Ratio of 4-O-Methyl Gallic Acid to Gallic Acid Concentration in Blood and Urine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (hour)</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portal vein</td>
<td>0.16</td>
<td>0.32</td>
<td>0.33</td>
<td>0.46</td>
<td>0.56</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Inferior vena cava</td>
<td>0.34</td>
<td>0.50</td>
<td>0.47</td>
<td>0.63</td>
<td>0.62</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>n.d.</td>
<td>0.60</td>
<td>0.55</td>
<td>0.62</td>
<td>0.76</td>
<td>0.69</td>
<td></td>
</tr>
</tbody>
</table>

n.d.: not determined.

**DISCUSSION**

In this study the metabolic fate of orally administered gallic acid was investigated by HPLC and the main metabolite was found to be 4-O-methyl gallic acid in peripheral blood and urine. Similar results were obtained by intraperitoneal and intravenous administration (data are not shown). After gallic acid was orally administered, it was absorbed fairly quickly and reached a maximum concentration (about 12 μg/ml) at 15 min in portal blood. On the other hand, 4-O-methyl gallic acid was also detected at about half the concentration of gallic acid in portal blood, suggesting the possibility of enterohepatic circulation or metabolism in the alimentary tract. Booth reported that when gallic acid was incubated with rat and rabbit liver slices in bicarbonate buffer (pH 7), 4-O-methyl gallic acid was detected in the medium. We also found 4-O-methyl gallic acid, but not gallic acid, in liver homogenate prepared after sufficient perfusion. These results suggest that liver metabolizes gallic acid into 4-O-methyl gallic acid, and that gallic acid is methylated as soon as it is taken up.

Analysis of metabolites in urine indicated that large amount of gallic acid and 4-O-methyl gallic acid were excreted into urine by 3 h after oral administration and that the ratio of gallic acid to 4-O-methyl gallic acid in urine was different from that in blood, indicating the possibility that 4-O-methyl gallic acid was preferentially excreted into urine relative to gallic acid. The concentration of gallic acid in portal blood was higher for 6 h than in inferior vena cava blood, suggesting that the absorption of gallic acid in the alimentary tract was prolonged, which maintains high gallic acid concentration in portal blood. In contrast, the blood level of gallic acid after intravenous or intraperitoneal administration declined rapidly as compared with oral administration, and a high level in portal blood was not obtained. However, the ratio of 4-O-methyl gallic acid to gallic acid was quite similar, regardless of the route of administration.

We previously reported that gallic acid shows cytotoxic activity against cancer cells with higher sensitivity than normal cells, whereas 4-O-methyl gallic acid did not. Taking into consideration that a relatively high concentration of gallic acid is present in portal blood and remains longer when gallic acid is administered orally, rather than intravenously or intraperitoneally, even though about 70% of gallic acid is detoxified by conversion into 4-O-methyl gallic acid, the cytotoxic activity shown by gallic acid should be more effective in the liver by oral administration than by the other administration method. The route of administration is an important factor that significantly influences efficacy of anti-cancer drugs. In addition, reducing pain or nuisance accompanied
by administration is another factor to be considered. In this respect, an orally applicable anticancer drug is an ideal one and gallic acid may be a candidate for such a drugs, as evidenced by the data presented.

In the present study we first determined the structure of the gallic acid metabolite in blood as 4-O-methyl gallic acid after oral administration and then examined the behavior of metabolites in blood in detail. However, we did not detect pyrogallol as a metabolite in blood or urine, as reported earlier. In these studies, the main metabolites of gallic acid in urine were determined by thin layer chromatography, but the structures were not properly determined. In addition, it took many hours to collect urine in unstable conditions, which suggests the possibility that decomposition of gallic acid occurred, resulting in the formation of pyrogallol. In conclusion, this study revealed that oral administration is suitable to gain a relative high concentration of gallic acid in blood, thus suggesting that oral administration of gallic acid would be suitable route to treat hepatic cancer.

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REFERENCES