Pharmacological Properties of Traditional Medicines. XXII. 1) Pharmacokinetic Study of Mulberroside A and Its Metabolites in Rat

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To identify the active components in Mori Cortex (Chinese medicine), Mori Cortex extracts were administered orally to rats, and then blood plasma, urine, and bile were analyzed. The results showed only a few remaining metabolites of mulberroside A. Consequently, to clarify the transitional properties of mulberroside A derivatives to tissues and the in vivo abundance ratio of mulberroside A compounds, the pharmacokinetics of mulberroside A derivatives were investigated in this study. When Mori Cortex extracts were administered orally, mulberroside A was only detected in small quantities in the plasma, and its bioavailability was about 1%. This is due to the first pass effect, by which most mulberroside A was converted into oxyresveratrol and transported into the circulating blood, and its absorption ratio was estimated at about 50%. Oxyresveratrol was also found to be transported to tissues at high rates. As a result, when analyzing the pharmacological activity of the Mori Cortex in vitro, it is more useful to study oxyresveratrol than mulberroside A.

Key words mulberroside A; oxyresveratrol; metabolite; Mori Cortex; pharmacokinetic; HPLC

Mori Cortex (桑白皮) is a dried root bark of Morus alba L. (Moraceae), and is found in Gokoto, Sel Eachito, Kyousan, and other traditional Chinese medicines. Gokoto is made by mixing Mori Cortex in Makyoukansekiito, and is used as an antitussive agent to treat severe asthma attacks. However, the active components of Mori Cortex have not yet been identified.

In most cases, traditional Chinese medicine is administered orally, and needs to be absorbed into the body in order to exhibit its biological effects. By orally administering the extracts of traditional Chinese medicine, and then identifying and verifying the compounds found in plasma, we have been determining the active components of traditional Chinese medicines. These biopharmaceutical studies further clarify the results of pharmacological studies on the active components of crude drugs, and play an important role in the field of pharmaceuticals. So far, we have been successful in obtaining some notable results.

Therefore, using the same methodology, we sought to determine the active components of Mori Cortex. After orally administering Mori Cortex extracts (MCE) to rats, the metabolites of mulberroside A were identified in the plasma, urine, and bile, then mostly mulberroside A was transported to body. In this study, before conducting detailed analyses on the pharmacology of mulberroside A and its aglycons, the pharmacokinetics of mulberroside A were investigated, producing some interesting findings.

MATERIALS AND METHODS

Crude Drug Mori Cortex, the dried root bark of Morus alba L., of Japanese Pharmacopoeia XII quality, was purchased from Tochimoto Co., Ltd. (Japan), in 1994.

Chemicals Mulberroside A (M-1)6 and oxyresveratrol (M-3)7 were isolated from Mori Cortex. Oxyresveratrol 2,3-di-O-β-D-glucuronide (M-2) and oxyresveratrol 2-α-β-D-glucuronide-3'-α-sulfate (M-4) were isolated from rat bile according to a previous report.11 These compounds were purified and used.

Preparation of MCE Mori Cortex (100 g) was added to 21 of distilled water, and the whole was boiled until the volume decreased to 1/2 of the original volume. Then, the extract was filtered through 5-layer gauze to give a filtrate (MCE), which was freeze-dried. Two hundred mg of the freeze-dried MCE corresponds to 1 g of the crude drug. This freeze-dried MCE was stored at 4 °C until use.

Animals Male Wister rats (6 weeks old, 150–180 g) were purchased from Shizuoka Laboratory Animal Center Co., Ltd. (Hamamatsu, Japan). The animals were kept in a breeding room (temperature: 24 ± 1 °C, humidity: 50–55%, 12–40 cycles) for 7 d before the start of the experiments. Tap water and normal food were given ad libitum. They were fasted for about 24 h before the start of the experiment. The freeze dried MCE and mulberroside A were orally administered to rats at a dose of 1.5 g/kg and 50, 100 mg/kg in the form of an aqueous suspension or solution, respectively. Mulberroside A was intravenously administered to rats at a dose of 25 and 50 mg/kg in the form of a saline solution. Oxyresveratrol was intravenously administered to rats at a dose of 10 mg/kg in the form of a 20% methanol aqueous solution.

Preparation of Plasma Sample for HPLC Analysis Freeze dried MCE and mulberroside A were orally administered to rats. After a designated time period, the cervical artery was cut under ether anesthesia and blood was collected. The blood was immediately centrifuged at 3000 rpm for 10 min at room temperature. Then, 2 ml of plasma was taken and added to 10 ml of methanol, and the mixture was stirred well. The mixture was ultrasonically vibrated for 1 min and centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was evaporated to dryness below 40 °C in vacuo. The residue was dissolved in 0.2 ml of methanol and filtered through

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a 0.45 μm filter for 3D-HPLC analysis; 50 μl of the filtrate was injected.

**Preparation of Urine Sample for HPLC Analysis** Each rat was kept in one metabolic cage (Sugiyamagen Co., Ltd.) under the same conditions as breeding. The freeze-dried MCE and mulberroside A were orally administered to rats. A urine sample was collected from 0 to 48 h after administration. The urine was kept at -10°C. The mixture was evaporated to dryness in vacuo below 40°C. Then, the residue was dissolved in 2 ml of methanol and filtrated through a 0.45 μm membrane filter, and 50 μl of the filtrated solution was injected into HPLC.

**Preparation of Bile Sample for HPLC Analysis** A polyethylene tube was inserted into the rat bile duct under pentobarbital anesthesia. Mulberroside A or oxyresveratrol were administered to rats, under the same conditions used for the urine sample. A bile sample was collected from the duct for a designated time period after administration. The bile was adjusted to 5 ml of methanol and filtrated through a 0.45 μm filter, then 20 μl of the solution was injected into HPLC.

**HPLC Analysis** 3D-HPLC was carried out on a Waters 510E gradient system equipped with a Waters 991 J photodiode array detector and its data processor. HPLC was performed under the following conditions. The column was Inertsil ODS-2 (4.6 × 250 mm, GL Science, Inc.). Column temperature was 40°C. Flow rate was 1 ml/min. Wavelength was 200–400 nm. Solvent A and B were 0.1% acetic acid water solution and 0.1% acetic acid of acetonitrile solution, respectively. The mobile phase for the plasma sample was A/B = 85/15. The mobile phase for urine and bile samples started from A/B = 90/10 and went to A/B = 66/34 for 60 min.

**Calibration Curve** Mulberroside A was dissolved in acetic acid–acetonitrile–water (0.1:15:85) and severely diluted to give solutions for the calibration curve of the drug in plasma. Mulberroside A, oxyresveratrol, oxyresveratrol 2,3′-o-β-D-glucuronic acid and oxyresveratrol 2-β-D-glucuronic acid–3′-o-sulfate were dissolved in acetic acid–acetonitrile–water (0.1:10:90) and severely diluted to give solutions for the calibration curves of the drugs in bile and urine.

**Kinetic Study** Plasma concentration–time data after the administration of mulberroside A was analyzed by a non-linear least squares regression program, MULTI.

**RESULTS**

**Preparation of Standard Curves and Recovery Tests** The standard curve for mulberroside A in plasma was drawn, and it was found that there was a strong linear relationship with a correlation coefficient value of 0.999 was obtained between 0.005 and 10.0 μg/ml (plasma concentration). Using the least-squares method, a regression equation of $y=0.08123x+2.568$ (x: mulberroside A peak area, y: concentration of mulberroside A in plasma) was obtained. Recovery rates were 94.2 to 99.7%, and reproducibility was excellent (Table 1).

<table>
<thead>
<tr>
<th>Added (μg/ml)</th>
<th>Found (μg/ml)</th>
<th>Accuracy ± C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0237</td>
<td>—</td>
</tr>
<tr>
<td>0.1000</td>
<td>0.1179</td>
<td>94.2 ± 6.7</td>
</tr>
<tr>
<td>1.0000</td>
<td>1.0204</td>
<td>99.7 ± 4.3</td>
</tr>
<tr>
<td>5.0000</td>
<td>4.8971</td>
<td>97.5 ± 4.1</td>
</tr>
</tbody>
</table>

The plasma was collected at 30 min after administration of mulberroside A (100 mg/kg). a: C.V.: coefficients of variation.

<table>
<thead>
<tr>
<th>Sample M-2</th>
<th>Added (μg)</th>
<th>Found (μg)</th>
<th>Accuracy ± C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31.8</td>
<td>—</td>
<td>94.7 ± 2.1</td>
</tr>
<tr>
<td>20.0</td>
<td>213.2</td>
<td>—</td>
<td>99.7 ± 4.4</td>
</tr>
<tr>
<td>200.0</td>
<td>206.4</td>
<td>—</td>
<td>98.4 ± 2.3</td>
</tr>
<tr>
<td>400.0</td>
<td>207.4</td>
<td>—</td>
<td>99.7 ± 4.4</td>
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</tbody>
</table>

Table 2. Accuracy of the Determination of Mulberroside A in Rat Bile after Oral Administration of Mulberroside A (n = 5)

<table>
<thead>
<tr>
<th>Sample M-2</th>
<th>Added (μg)</th>
<th>Found (μg)</th>
<th>Accuracy ± C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-2</td>
<td>0</td>
<td>143.3</td>
<td>95.9 ± 6.1</td>
</tr>
<tr>
<td>200.0</td>
<td>2067.7</td>
<td>—</td>
<td>96.2 ± 4.6</td>
</tr>
<tr>
<td>2000.0</td>
<td>2067.7</td>
<td>—</td>
<td>96.2 ± 4.6</td>
</tr>
</tbody>
</table>

Table 3. Accuracy of the Determination of Mulberroside A and its Metabolites in Rat Urine after Oral Administration of Mulberroside A (n = 5)

The results were obtained (Tables 2 and 3).

**Determination of Mulberroside A in Plasma** Figure 1 shows chronological changes in the concentration of mulberroside A in plasma. When 25 or 50 mg/kg of mulberroside A was administered intravenously, mulberroside A in plasma disappeared with a half-life of about 30 min. Also, when 100 mg/kg of mulberroside A was

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M.1, mulberroside A; M.2, oxyresveratrol 2,3′-o-β-D-glucuronic acid, M.3, oxyresveratrol. The urine was collected from 0 to 48 h after the administration of mulberroside A (100 mg/kg).
administered orally, absorption occurred within 60 min, and it disappeared from the plasma in the same manner observed with the intravenous administration. However, when 1500 mg/kg of the extract (equal to 50 mg/kg of mulberroside A) was administered orally, the half-life was longer.

**Determination of Metabolites in Bile** Table 4 shows the amount of biliary excretion per kg of body weight and shifts over time. The excretion amount was calculated based on mulberroside A. When 100 mg/kg of mulberroside A was administered orally, mulberroside A could hardly be detected in plasma after 3 h, whereas the excretion of metabolites to bile reached a maximum in 6 to 9 h after administration and continued for more than 24 h. Mulberroside A was most frequently excreted as diglucuronide; oxyresveratrol was also excreted beyond 24 h, and there were no marked shifts in the excretion ratios of metabolites. Furthermore, when mulberroside A was administered intravenously, 63.4% of the excrements were mulberroside A between 0—3 h after administration, and 27% between 3 to 6 h after administration.

**Determination of Metabolites in Urine** Figure 2 shows the excretion amount of urinary excrements and its ratios. When the MCE was administered orally, the excretion amount of metabolites in urine was about 4%, compared to about 7% when mulberroside A was administered orally. The main component of the excrements was oxyresveratrol (an aglycon of mulberroside A), and some diglucuronide compounds of oxyresveratrol were also excreted. When mulberroside A was administered intravenously, mulberroside A was excreted, so intravenous mulberroside A clearly follows a different excretion pathway compared with oral administration.

On the other hand, when oxyresveratrol was administered intravenously, its excretion was unchanged from that after oral administration.

**Kinetic Analysis** Table 5 shows the kinetic parameters of mulberroside A and its metabolites, as calculated from the plasma concentration and urine excretion amounts following the oral and intravenous administration of mulberroside A.

The distribution volume of mulberroside A was about 8 l/kg. Also, the half-life of mulberroside A in plasma was about 30 min, which did not coincide with the half-life values for mulberroside A in urine or bile. Its bioavailability was less than 1% when calculated based on the ratios of the plasma concentration–time curve
Table 5. Pharmacokinetic Parameters for Mulberroside A to Rat

<table>
<thead>
<tr>
<th></th>
<th>Dose (mg/kg)</th>
<th>$V_d$ (l/kg)</th>
<th>$T_{1/2}$ (h)</th>
<th>$AUC$ (mg/l-h)</th>
<th>$E_A$ (%)</th>
<th>$E_BA$ (%)</th>
<th>$AR$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>p.o.</td>
<td>1500</td>
<td>—</td>
<td>0.032</td>
<td>4.2</td>
<td>0.75</td>
<td>27.8</td>
</tr>
<tr>
<td>M-1</td>
<td>p.o.</td>
<td>100</td>
<td>—</td>
<td>0.050</td>
<td>7.2</td>
<td>0.60</td>
<td>47.7</td>
</tr>
<tr>
<td>M-1</td>
<td>i.v.</td>
<td>50</td>
<td>7.19</td>
<td>0.024</td>
<td>7.5</td>
<td>0.56</td>
<td>49.7</td>
</tr>
<tr>
<td>M-3</td>
<td>i.v.</td>
<td>25</td>
<td>9.90</td>
<td>1.9</td>
<td>23.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>15.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$E_A$, excretion amount; $E_BA$, extent of bioavailability; $AR$, absorption ratio; M-1, mulberroside A; M-3, oxyresveratrol.

(AUC) between oral and intravenous administrations. Furthermore, based on the ratio of the urinary excretion amount of mulberroside A in oral administration and that of oxyresveratrol in intravenous administration, the total absorption ratio of mulberroside A and its derivatives was calculated to be about 50%.

DISCUSSION

In most cases, traditional Chinese medicine is administered orally, so in order to identify the true active components of Mori Cortex, as a first step, its water extracts were administered orally, and the constituents of plasma, urine, and bile were analyzed. Tests detected the presence of mulberroside A in plasma, and its metabolites in urine and bile. If the effective constituents of Mori Cortex are investigated by an in vitro test, it would be necessary to conduct biopharmaceutical studies of mulberroside A and its metabolites. Therefore, mulberroside A was administered orally and intravenously, and oxyresveratrol was administered intravenously to measure kinetic parameters by measuring the plasma concentrations and urinary and biliary excretion amounts of mulberroside A and its metabolites.

The half-life of mulberroside A was about 30 min, a value that did not coincide with the excretion kinetics of mulberroside A to urine and bile. This time difference suggests that once inside the body, mulberroside A exists in the form of its derivatives. Also, when its bioavailability is calculated based on the ratios of the AUC between intravenous and oral administrations, a value of less than 1% was obtained, further indicating that mulberroside A is not transported unchanged within the body.

However, when mulberroside A was administrated orally, the main component of urinary excrements was its aglycon, oxyresveratrol, and when mulberroside A was administered intravenously, mostly mulberroside A was excreted. When oxyresveratrol was administered intravenously, oxyresveratrol was excreted unchanged. Also, oxyresveratrol was excreted to bile for more than 24 h, and the ratios of compounds that were excreted to bile did not change markedly. Therefore, it is predicted that oxyresveratrol metabolism is the rate-determining step of mulberroside A metabolism. Based on these findings, it is hypothesized that when mulberroside A is administered orally, due to a first pass effect, mulberroside A exists in the form of oxyresveratrol inside the body.

The total absorption amount of mulberroside A and its derivatives, calculated from the ratio between urinary excretion amounts following the oral administration of mulberroside A and the intravenous administration of oxyresveratrol, was about 50%. Therefore, orally administered mulberroside A was metabolized in the liver or digestive tract, and about 1% of mulberroside A and about 50% of its metabolites were transported to the circulating blood, then these absorbed components were excreted to bile and urine in a ratio of 3:1.

However, when mulberroside A was administered orally, oxyresveratrol could not be detected in the plasma. Furthermore, when oxyresveratrol was administrated intravenously, only extremely small amounts of oxyresveratrol were detected in plasma, even 5 or 10 min after administration. Moreover, since the distribution volume of mulberroside A in plasma was about 8 l/kg, it is thought that the distribution volume of oxyresveratrol is extremely large, and that oxyresveratrol is easily transported to tissue.

Thus, mulberroside A satisfied the conditions necessary to be considered an active component of the Mori Cortex, and it is thought that mulberroside A is a strong candidate for being the true active component of Mori Cortex. However, when Mori Cortex extracts are administered orally, there is a good possibility that the main component, mulberroside A, exists as a prodrug, expressing pharmacological effects after being converted to oxyresveratrol in the body. In effect, we believe it is more effective to analyze oxyresveratrol than mulberroside A when studying the pharmacological effects of Mori Cortex in vitro.

In the past, experimentally isolated components from a crude drug were analyzed directly in vitro in many cases, so information regarding true active components was minimal and only in vivo physiological reactions were measured in experiments. Thus, by conducting biopharmaceutical studies of the active components of traditional Chinese medicine, the relationship between the components of traditional Chinese medicine and the true active components in the body can be more clearly defined; this in turn strengthens the validity of pharmacological analyses. We believe that the same kind of analyses as those conducted in this study are necessary when identifying the active components of traditional Chinese medicines in the future.

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