Introduction

Magnolol (M; Fig. 1) is a polyphenolic lignan in the bark of *Magnolia officinalis* Rehder & E. Wilson (Magnoliaceae), which is a frequently prescribed Chinese herb for the treatment of anxiety, fever, headache, and neurosis [1]. Various beneficial activities of M have been reported, including anti-inflammation [2–5], anticancer [6–9], antibacterial [5,10,11], antispasmodic [12], and antidepressive effects [13,14]. Most of the in vitro bioactivities of M reported were mainly of the parent form. Nonetheless, in the analysis of the biological fate of polyphenols, it has been increasingly recognized that their parent forms were generally not present in circulation [15]. Therefore, whether the in vitro bioactivities of M could predict the in vivo effects and what are the actual molecules working in vivo remain unanswered. Even if the intravenous pharmacokinetics of M and oral pharmacokinetics of [ring-14C] labeled M have been previously reported [16,17], more detailed information of the pharmacokinetics and tissue distribution of M and its metabolites is still lacking. Therefore, this study investigated the pharmacokinetics and tissue distribution of M and magnolol sulfates/glucuronides (M S/G) following intravenous and oral administrations in rats.

Materials and Methods

Plant material

The crude drug of the bark of *Magnolia officinalis* was purchased from a Chinese drugstore in Taichung, Taiwan. The origin was identified by Dr. Yu-Chi Hou by microscopic examination, and a voucher specimen (CMU-P-1905-03) was deposited in the Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung, Taiwan.
Chemicals
Butyl paraben, cremophor EL, 1,2-propanediol, β-glucuronidase (type B-1 from bovine liver, containing 1 240 000 units/g of β-glucuronidase) and sulfatase (type H-1 from *Helix pomatia*, containing 14 000 units/g of sulfatase and 498 800 units/g of β-glucuronidase) were purchased from Sigma. L(+)-ascorbic acid and ortho-phosphoric acid were obtained from Riedel-deHaën AG. Acetonitrile, ethyl acetate, and methanol were obtained from J.T. Baker, Inc. Magnolol was obtained from Wako (purity 99%). n-Hexane was purchased from ALPS Chemical, Ltd. Sodium acetate was obtained from Kohusan Chemical Works, Ltd. Milli-Q plus water (Millipore) was used for all processes.

Instrumentation
The high-performance liquid chromatography (HPLC) apparatus included a pump (LC-6AD; Shimadzu), an UV spectrophotometric detector (SPD-6A; Shimadzu), a chromatopac (C-R6A; Shimadzu), and an autosampler (Series 200; Perkin Elmer). RP-18e column (LiChroSpher, 250 × 4.0 mm) was used with a prefilter (Isolation Technologies).

Animals and drug administration
All animal experiments adhered to “The Guidebook for the Care and Use of Laboratory Animals” published by the Chinese Society of Animal Science, Taiwan, ROC. The animal protocol (95-159-D) was approved by the Institutional Animal Care and Use Committee of the China Medical University, Taiwan. Male Sprague-Dawley rats (350–450 g) were supplied by BioLASCO and kept at least 2 weeks under a conditioned environment with free access to food and water. Before the experiment, the rats were fasted overnight but drinking water was allowed *ad libitum*. Food was supplied 3 h after M dosing. M solution was prepared by dissolving M in a vehicle composed of equal weight of 1,2-propanediol and cremophor EL to afford a concentration of 20 mg/mL. For the pharmacokinetic study, five rats were given an intravenous bolus of M at 20 mg/kg; in addition, rats were orally given a single dose (n = 5) and seven doses (n = 6) of M at 50 mg/kg by gastric gavage. For the tissue distribution studies, three rats were orally given 50 mg/kg of M thrice daily for seven doses prior to the sacrifice for tissue collection.

Blood specimen collection
In the pharmacokinetic study, blood samples (0.7 mL) were collected via cardiac puncture at 5, 10, 15, 20, 30, 45, 60, 120, 240, and 480 min after intravenous dosing and at 5, 10, 15, 30, 60, 120, 240, and 480 min after oral administration. In the tissue distribution study, a blood sample was withdrawn at 30 min after the 7th dose of oral M. The sera were collected by centrifugation at 10 000 g for 15 min to obtain the supernatants and stored at −30°C until analysis.

Tissue collection and pretreatment
At 30 min after the 7th dose of M, rats were sacrificed by inhaling CO₂ and systemically perfused with cold normal saline. Then, the organs including the brain, liver, lung, heart, and kidney were removed, blotted dry with filter paper and accurately weighed. The tissues were homogenized with normal saline (700 mg/mL), and the homogenates were stored at −30°C until analysis.

Quantitation of M in serum and tissue homogenates
To determine the serum concentration of M, 100 μL of serum was added to 100 μL of acetate buffer (pH 5.0), 50 μL of ascorbic acid (200 mg/mL), and 50 μL of 0.1 N HCl, then partitioned with 300 μL of ethyl acetate containing 5.0 μg/mL of butyl paraben as the internal standard. After centrifugation at 10 000 g for 15 min, the supernatant was dried under nitrogen gas and reconstituted with an appropriate volume of acetonitrile before HPLC analysis. For the determination of M concentration in various tissue homogenates, 500 μL of homogenate was added to 100 μL of acetate buffer (pH 5.0), 100 μL of ascorbic acid (200 mg/mL), and 20 μL of 0.1 N HCl, then partitioned with 700 μL of ethyl acetate containing 0.5 μg/mL of butyl paraben as the internal standard. The later procedures followed that of serum samples. For HPLC analysis of both serum and tissues, the mobile phase used was acetonitrile/0.1% ortho-phosphoric acid (48:52), and it was run isocratically. The flow rate was 1.0 mL/min with the detection wavelength set at 290 nm.

Quantitation of M S/G in serum and tissue homogenates
For serum analysis, 100 μL of serum, 100 μL of sulfatase solution (containing 10 units of sulfatase and 210 units of β-glucuronidase in pH 5.0 acetate buffer), and 50 μL of ascorbic acid (200 mg/mL) were added into a light protected test tube and incubated at 37°C for 120 min, which had been determined by a preliminary study for reaching optimum hydrolysis. For tissue analysis, 500 μL of various tissue homogenates, 100 μL of sulfatase solution (containing 10 units of sulfatase and 210 units of β-glucuronidase in pH 5.0 acetate buffer), and 100 μL of ascorbic acid (200 mg/mL) were added into a light protected tube and incubated at 37°C for 120 min, which had been determined by a preliminary study. The concentration of M S/G was calculated from the total M concentration after hydrolysis minus that before hydrolysis. The later procedures followed that described above for the quantitation of M in tissue.

Quantitation of MG in serum and tissue homogenates
For the analysis of serum, 100 μL of serum, 100 μL of β-glucuronidase solution (containing 10 units of β-glucuronidase in pH 5.0 acetate buffer), and 50 μL of ascorbic acid (200 mg/mL) were added into a light protected test tube and incubated at 37°C for 60 min, which had been determined by a preliminary study for reaching optimum hydrolysis. For tissue analysis, 500 μL of homogenates, 100 μL of β-glucuronidase solution (containing 100
units of β-glucuronidase in pH 5.0 acetate buffer), and 100 µL of
ascorbic acid (200 mg/mL) were added into a light protected tube
and incubated at 37°C for 120 min, which had been determined
by a preliminary study. The concentration of MG was calculated
from the total M concentration after hydrolysis minus that before
hydrolysis. The later procedures followed that described above
for the quantitation of M in tissue.

Validation of assay methods
The precision and accuracy of the assay methods were evaluated
by intraday and interday analysis of triplicates at concentrations
of 0.5, 3.1, and 25.0 µg/mL of M in serum, and 0.16, 0.63, and
2.5 µg/mL of M in various tissue homogenates over a period of
days. Lower limit of quantitation (LLOQ) represents the
lowest concentration of analyte in a sample that can be deter-
m定了 with acceptable precision and accuracy, whereas limit of
detection (LOD) represents the lowest concentration of analyte
in a sample that can be detected (with S/N > 3).

Calculation of pharmacokinetic parameters
and statistical analysis
Pharmacokinetic parameters were calculated by a noncompartment
model of WinNonlin (version 1.1 SCI software; Statistical
Consulting, Inc). The peak serum concentration (C_{max}) and the
time to peak concentration (T_{max}) were calculated based on ex-
perimental measurements. The areas under the curves (AUC_{0-\text{t}})
from time zero to last were calculated by the trapezoidal rule.
The oral absolute bioavailability (F) of M was calculated by the
following equation:

$$F = \frac{\text{AUC}_{0-\text{t}} \cdot \text{Dose}_{PO}}{\text{AUC}_{0-\text{t}} \cdot \text{Dose}_{IV}}$$

One-way ANOVA with Scheffe’s test was used for statistical com-
parisons.

Supporting information
The method of magnolol isolation and validation of the analytical
methods are available as Supporting Information.

Results

The calibration curve of M in the range of 0.13–5.00 µg/mL in se-
rum showed good linearity (r > 0.99). The precision evaluation
showed that all coefficients of variation were below 8.6%, and
the accuracy analysis demonstrated that the relative errors to
the true concentrations were below 8.0%. The recoveries of M
from serum were 86.7–96.5% at 0.5, 5.0, and 20.0 µg/mL. The
LLOQ and LOD of M in serum were 0.13 and 0.06 µg/mL, respec-
tively.

For tissue analyses, good linearities (r > 0.99) were obtained in
the ranges of 0.16–2.5 µg/mL of M in the brain homogenate and
0.08–2.5 µg/mL in the homogenates of the liver, kidney, lung,
and heart. The precision evaluation showed that all coefficients
of variation were below 17.2%, and the accuracy analysis deter-
minded that the relative errors to the true concentrations were
below 12.5%. The recoveries of M from the brain, liver, lung, heart,
and kidney were 106.4–108.5%; 104.3–113.7%; 85.1–106.4%;
95.2–113.7%; and 81.6–98.6%, respectively, at 0.2, 0.6, and
2.5 µg/mL. The LLOQ of M was 0.16 µg/mL in the brain homoge-
nate and 0.08 µg/mL in other tissues. The LOD was 0.04 µg/mL in
all tissue homogenates.

The mean serum concentration-time profiles of M, MG, and M S/G
after an intravenous bolus of M are shown in Fig. 2. The
results showed that M S/G emerged instantaneously and the serum
profiles of M, MG, and M S/G were largely superposable. The
pharmacokinetic parameters of M, MG, and M S/G are listed in
Table 1. The AUC_{0-\text{t}} of M, MG, and M S/G were comparable.

**Table 1** Pharmacokinetic parameters of magnolol (M), magnolol glucuro-
nides (MG), and magnolol sulfates/glucuronides (M S/G) after intravenous bolus
(20 mg/kg, n = 5), oral administration of a single dose (n = 5), and seven
doses (n = 6) of 50 mg/kg of magnolol to rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameters</th>
<th>T_{max} (min)</th>
<th>C_{max} (nmol/mL)</th>
<th>AUC_{0-480} (nmol·min/mL)</th>
<th>MRT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous bolus</td>
<td>M</td>
<td>230.0 ± 85.1</td>
<td>1.5 ± 0.2a</td>
<td>466.9 ± 47.4b</td>
<td>240.4 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>MG</td>
<td>132.5 ± 78.4</td>
<td>7.3 ± 1.2a</td>
<td>2138.6 ± 494.7b</td>
<td>235.4 ± 13.5</td>
</tr>
<tr>
<td></td>
<td>M S/G</td>
<td>136.7 ± 46.7</td>
<td>10.6 ± 2.7a</td>
<td>2933.5 ± 761.7b</td>
<td>230.9 ± 3.2</td>
</tr>
<tr>
<td>Oral single dose</td>
<td>M</td>
<td>18.3 ± 3.8</td>
<td>2.6 ± 0.5a</td>
<td>228.5 ± 23.2a</td>
<td>82.2 ± 12.9a</td>
</tr>
<tr>
<td></td>
<td>MG</td>
<td>18.0 ± 3.0</td>
<td>7.5 ± 1.4b</td>
<td>1244.5 ± 186.9b</td>
<td>266.4 ± 52.0b</td>
</tr>
<tr>
<td></td>
<td>M S/G</td>
<td>51.0 ± 18.7</td>
<td>7.3 ± 1.2b</td>
<td>1035.0 ± 118.7b</td>
<td>146.2 ± 8.4b</td>
</tr>
<tr>
<td>Oral seven doses</td>
<td>M</td>
<td>230.0 ± 85.1</td>
<td>1.5 ± 0.2a</td>
<td>466.9 ± 47.4b</td>
<td>240.4 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>MG</td>
<td>132.5 ± 78.4</td>
<td>7.3 ± 1.2a</td>
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</tr>
</tbody>
</table>

Values are means ± SE. C_{max}: maximum serum concentration; AUC_{0-480}: area under
serum concentration-time curve to the last time (480 min); MRT: mean residence
time. Means in a column without a common superscript differ, p < 0.05.

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in the blood rapidly after oral dosing, and the serum profile of M was much lower than that of M S/G. The pharmacokinetic parameters are listed in Table 1. After single-dose administration, the C_{max} and AUC_{0–480} of M S/G were significantly higher than those of M by 180% and 350%, respectively. After the 7th dose, the C_{max} and AUC_{0–480} of M S/G were significantly higher than those of M by 606% and 584%, respectively. Comparison of the C_{max} of M between two treatments showed that single-dose administration resulted in a higher C_{max} of M than the 7th dose by 73%. On the contrary, the AUC_{0–480} of M and M S/G after the 7th dose was significantly higher than those after a single dose by 104% and 183%, respectively.

The distribution of M, MG, and M S/G in various tissues after the 7th dose of M are shown in Table 1. Contrary to the finding in serum, the major molecule in each organ was M rather than M S/G. The concentration of M in the liver was the highest and those in the kidney, brain, lung, and heart were much lower and comparable. All organs assayed contained higher concentrations of M than serum. The liver also contained the highest concentration of M S/G than other organs and even higher than serum, whereas only traces of M S/G were present in the lung, heart, brain, and kidney. The concentrations of M S/G were comparable with those of MG in these organs.

Discussion

In this study, we established and validated the quantitation method of M in serum and various tissue homogenates. The analytical precision and accuracy as well as recoveries were satisfactory. Owing to the unavailability of authentic standards of MG and magnolol sulfates (MS), β-glucuronidase and sulfatase were used to hydrolyze them, and then the released M was determined. The commercial sulfatase (type H-1) used in this study contained not only sulfatase but also a considerable amount of β-glucuronidase. Therefore, both sulfates and glucuronides were hydrolyzed when treated with sulfatase. Through comparing the released amounts of M between treatments with sulfatase/glucuronidase and β-glucuronidase, the concentration of MS could be estimated.

A previous study reported the intravenous pharmacokinetics of M in rats, but only M had been quantitated [17]. In this study, we determined not only M but also M S/G. After an intravenous bolus, the serum profiles revealed that M was instantaneously metabolized by the liver into M S/G [18, 19]. Comparable AUC_{0–480} between MG and M S/G, shown in Table 1, indicated that the major conjugates in the circulation were MG whereas MS were negligible. The AUC_{0–480} of M and MG was comparable, implying that they were equally exposed to the vascular system.

When rats were orally administered with a single dose of M (50 mg/kg), both M and M S/G were found in the serum. Fig. 3A revealed that M was rapidly absorbed and metabolized extensively by conjugation reactions like other polyphenols [18–21]. The presence of a free form in serum is not quite usual among polyphenols, which were known to be exclusively metabolized during the first pass [22, 23]. The comparable AUC_{0–480} between MG and M S/G indicated that MG were the major conjugates. The significantly higher C_{max} and AUC_{0–480} of MG than M revealed that the major molecules in serum were MG. This finding was consistent with a previous study reporting that bile contained 48–49% of MG, 7% of M, and 2% of MS after oral administration...
of M to rats [16]. The absolute bioavailability of M was calculated as being only 4% which is similar to a previous study [24]. The low bioavailability might be partly explained by the high metabolism during the passage through the gut and liver [16]. If the AUCC0–480 of M S/G was added into the calculation, the oral bioavailability of M became 17.8%. The poor bioavailability of M might be accounted for by its low solubility in gastrointestinal juice.

In order to understand the steady-state pharmacokinetics of M, rats were orally given 50 mg/kg of M three times a day for 7 doses. Fig. 3B depicting the serum profiles after the 7th dose revealed an apparently different pattern from that in Fig. 3A. The concentrations of M appeared at a lower and stable level whereas those of M S/G were much higher and with three peaks at 15, 60, and 240 min, which was in agreement with a previous study suggesting enterohedetic circulation of M conjugates [16]. The AUCC0–T of M S/G was higher than that of MG by 58% although statistical significance was not reached (p = 0.08). In contrast to single-dose pharmacokinetics, we speculate that after repeated dosing, the glucuronidation of M might have been saturated and sullafation took a turn as an alternative metabolic pathway. In addition, serum specimens had been collected right before the 4th and 7th dose and quantitated. The concentrations of M S/G were 1.5 ± 0.3 and 3.4 ± 0.4 nmol/mL, respectively, whereas M was not detected. This evidence indicated that there was accumulation of M S/G but not M.

For tissue distribution analysis, in order to prevent the interference of residual blood, rats were systemically perfused with cool normal saline before organ collection. The results show that M was the major form in various organs, which was opposite to the finding in serum in which M S/G were predominant. We speculate that M S/G in the blood were hydrolyzed by β-glucuronidase or sulfatase in the surface cells of various organs and then permeated into the organs [25–27]. Among the assayed organs, comparable concentrations of M S/G and MG indicated that MG were the major conjugated metabolites. The liver contained the highest concentration of M and M S/G, which was in good agreement with the finding of a previous study using [ring-14C] labeled M [16]. Nevertheless, the previous study was not able to distinguish M from M S/G. In contrast, our study found that both M and MG were present in the liver, and M was the major form.

Despite the anti-hepatoma activity of M demonstrated by in vitro studies [6,8], the reported effective concentration (100 nmol/mL) may not be achievable in vivo based on the estimation from our pharmacokinetic findings. Besides, the liver also contained the highest concentration of MG among the studied organs. In the past, the conjugated metabolites were thought to be inactive and eliminated as the final products of drugs from the body. However, the conjugated metabolites of polyphenols increasingly attracted the interest of researchers. For instance, the paracogenic effect of morphine 6-glucuronide was found to be more potent than the one of morphine, and a clinical trial of morphine 6-glucuronide is ongoing [28,29]. In addition, the glucuronides of morin and quercetin showed potential bioactivities [30–33]. Till now, most in vitro bioactivity studies investigated M only; rather than M S/G [6,7,9,11]. Given that abundant M S/G were present in the bloodstream and liver, the bioactivities of M S/G in the vascular system and liver warrant future studies.

In summary, M S/G were the major forms in circulation, whereas M was predominant in the liver, kidney, brain, lung, and heart after oral administration of M. Among the tissues, the liver contained the highest concentrations of M and MG.

Acknowledgements

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Conflict of Interest

The authors report no conflict of interest.

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