Berberine is an isoquinoline alkaloid isolated from several Chinese herbal medicines such as rhizoma coptidis, cortex phellodendri, and caulis mahoniae. Its chemical structure is 5,8-dihydro-9,10-dimethoxy-
-quinolizine. Berberine exhibits a wide variety of bioactivities such as antidiarrheic (Sack and Frowlich, 1982; Taylor and Greenough, 1989), antimicrobial (Yan et al., 2008), hypolipidemic (Kong et al., 2004; Doggrel, 2005; Brusq et al., 2006; Cicero et al., 2007), hypoglycemic (Yin et al., 2002; Pan et al., 2003; Zhou et al., 2007; Turner et al., 2008; Yin et al., 2008), antiarrhythmic (Wang et al., 1994; Lau et al., 2001), anticancer (Inoue et al., 2005; Lanvers-Kaminsky et al., 2006; Lin et al., 2006, 2007; Pyunch et al., 2007; Serafim et al., 2008; Yu et al., 2007), anti-inflammatory (Kuo et al., 2004; Lee et al., 2007), antiviral (Hayashi et al., 2007), antidepresant (Kulkarni and Dhir, 2007), and hepatoprotective (Zhang et al., 2008) effects. Its chloride salt has been used for several decades in clinical situations to treat gastroenteritis and secretory diarrhea in China. In recent years, berberine has also shown significant effects in treatment of diabetes mellitus (Ni, 1988), hyperlipemia (Kong et al., 2004), arrhythmia, and heart failure (Zeng and Zeng, 1999; Lau et al., 2001; Zeng et al., 2003). However, pharmacokinetic studies have indicated that berberine has poor oral bioavailability (Shen et al., 1993; Yu et al., 2000; Zuo et al., 2006), and a few of the metabolites have been identified in rats (Zuo et al., 2006) and in humans (Pan et al., 2002). To obtain more information about its metabolism to improve its clinical applications, we examined the bio-
transformation of berberine in rats and humans. In the present article we describe the isolation and identification of urinary metabolites of berberine in these two species.

Materials and Methods

Materials. Berberine chloride (purity > 99.5%) was supplied by the Northeast General Pharmaceutical Factory (Shenyang, China). Methanol was HPLC grade and water was double-distilled in our laboratory. All other reagents were of analytical grade and were purchased from Shenyang Chemical Company (Shenyang, China). Normal-phase and reverse-phase preparatory thin-layer chromatography was performed using products from Merck (Darmstadt, Germany). Macroporous resin D101 was purchased from the Chemical Plant of Nankai University (Tianjin, China) and Diaion ion exchange resin HP20 from Mitsubishi Chemical Corporation (Tokyo, Japan). Sephadex LH-20 and ODS were obtained from Pfizer (Freiburg, Germany). Spots on chromatograms were detected with Dragendorff’s reagent (a solution of potassium bismuth iodide), which is usually used to visualize alkaloids that have been developed using thin-layer chromatography. Arylsulfatase (EC 3.1.6.1) and β-glucuronidase (EC 3.2.1.31) were purchased from Sigma-Aldrich (St. Louis, MO).

Subjects and Dosing Procedure. Eighty male Wistar rats, from 8 to 10 weeks old and weighing 200 to 250 g, were used in the study of the urinary metabolites of berberine chloride in rats. Rats were kept in the breeding room for 7 days before the study. Normal food and water were available at all times but were withdrawn 12 h before intragastric administration of berberine chloride. Each rat was given an oral dose (100 mg/kg b.wt.), of berberine chloride and urine samples were collected for 48 h.

For the study of the urinary metabolites of berberine chloride in humans, 12 healthy volunteers from 22 to 26 years of age and weighing 60 to 80 kg (all male) participated in this study. Subjects were judged to be in good health on the basis of a medical history, physical examination, and laboratory profiles that were obtained within a 2-week period before the start of the study. The study followed the 1964 Declaration of Helsinki for the ethical treatment of human subjects for biomedical research. Each subject was given a 300-mg oral
dose of berberine chloride, three times a day for 2 days, and urine was collected between 0 and 72 h.

**Isolation of Metabolites.** The urine samples (rat: 3800 ml; human: 16,000 ml) were chromatographed on macroporous adsorptive resin D101 and eluted with H2O and 95% EtOH. The 95% EtOH eluate was subjected to HP 20 column chromatography using an H2O-EtOH solvent system. The fractions eluted by 20 and 30% EtOH were then fractionated by chromatography on Sephadex LH-20 and RP-18 silica gel columns with an MeOH-H2O solvent system (0–30%). The fractions containing the metabolites underwent fractionation by Sephadex LH-20 and RP-8 and RP-18 silica-gel column chromatography with an MeOH-H2O solvent system (10–60%) and then were purified by preparative HPLC.

**Purification by HPLC.** Preparative HPLC was performed with an ODS column (X Terra RP18, 7 μm, 19 × 300 mm) in a Waters 600 liquid chromatograph equipped with a Waters 490 UV detector. The usual detection wavelength was 340 nm. Elution was carried out with MeOH-H2O at a flow rate of 10 ml/min. Elution with MeOH-H2O (5:15) yielded RM1 (2.3 mg). Elution with MeOH-H2O (16:84) yielded HM2 (6 mg), HM3 (4 mg), and HM7 (1 mg). Elution with MeOH-H2O (22:78) yielded HM1 (40 mg), HM4 (6.1 mg), HM5 (2.2 mg), and HM6 (1.2 mg). Elution with MeOH-H2O (55:45) yielded RM5 (10 mg).

**Spectroscopic Methods.** Electrospray ion trap mass spectrometry in multistage full scan mode was performed on a Bruker Esquire 2000 instrument operated in the both positive and negative ion modes, using nitrogen as the nebulizing and dry gas. The collision-induced dissociation of the quasi-molecular ion was detected by ESI-MS. Comparison of the 1H NMR data (Table 1) of the fragment ion [M − Cl] at m/z 338, suggesting that HM2 might be a glucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of HM2, in which the aglycone ion fragment at m/z 338 was detected by ESI-MS. Comparison of the 1H NMR data (Table 1) of the aglycone of HM2 with those of berberine indicated many similarities except for the loss of the characteristic methylene signal and the appearance of a new methoxyl signal.

**Results**

**Isolation and Structure Elucidation of Berberine Metabolites.** By means of macroporous adsorptive resin chromatography, open ODS column chromatography, and preparative-high-performance liquid chromatography, five metabolites (RM1–RM5) from rat urine and seven metabolites (HM1–HM7) from human urine were isolated after oral administration of berberine chloride, and their structures were identified on the basis of enzymatic hydrolysis, ESI-MS, 1H NMR, and NOESY spectra.

In the ESI-MS spectra (Fig. 1), removal of masses of 80 or 176 from the quasi-molecular ions [M − Cl]+ of the metabolites showed that they were sulfate or glucuronide conjugates. The evidence provides useful information to help in the structural identification of the berberine metabolites.

**Sulfate-Conjugated Metabolites.** HM1 (RM3) and HM5 were identified as demethyleneberberine-2-O-sulfate and jatrorrhizine-3-O-sulfate, respectively, by enzymatic hydrolysis and further comparison of MS and NMR data (Tables 1 and 2) with those in the literature (Pan et al., 2002).

HM6 (RM4) was obtained as yellow needle crystals, giving a positive reaction to Dragendorff’s reagent. ESI-MS of HM6 gave the quasi-molecular ion [M − Cl] at m/z 404 and [M − Cl + Na − H]+ at m/z 426. Bombardment of m/z 426 afforded a fragment ion [M − Cl + Na − H − SO4] at m/z 346. In combination with enzymatic hydrolysis data, HM6 was deduced to be a sulfate-conjugated metabolite.

In the 1H NMR spectrum, the skeleton proton signals (Table 2) of the parent drug remained except for those of the substituent groups. The linked positions of two methoxyl groups were established by the NOESY spectrum: the methoxyl signal at δ4.24 correlated with that of H-8 at δ9.83 and the methoxyl group at δ9.95 correlated with that of H-1 at δ7.72, indicating that two methoxyl groups were linked at C-9 and C-2. Thus, the sulfate group must be linked to C-3 or C-10. Furthermore, 9-OCH3 and H-11 shifted downfield by 0.1 ppm, and H-4 hardly shifted at all, so we concluded that the sulfate group was located at C-10. Thus, HM6 was identified as 3,10-dimethylpalmitine-10-O-sulfate.

**Glucuronide-Conjugated Metabolites.** HM2 was obtained as a yellow powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of HM2 gave the quasi-molecular ion [M − Cl] at m/z 514. Bombardment of 514 afforded the fragment ion [M − Cl − C6H8O6] at m/z 338, suggesting that HM2 might be a glucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of HM2, in which the aglycone ion fragment at m/z 338 was detected by ESI-MS. Comparison of the 1H NMR data (Table 1) of the aglycone of HM2 with those of berberine indicated many similarities except for the loss of the characteristic methylene signal and the appearance of a new methoxyl signal.

The linked positions of the newly formed methoxyl group and the glucuronic acid moiety were established by the NOESY spectrum, in which H-1 (δ7.73) exhibited correlations with H-12 (δ7.89) and H-13 (δ9.04) correlated with that of H-1 at δ7.73. Therefore, the structure of HM2 was identified as jatrorrhizine-3-O-β-D-glucuronide.

HM3 was obtained as a yellow powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of HM3 gave the quasi-molecular ion [M − Cl] at m/z 498. Bombardment of 498 afforded the fragment ion [M − Cl − C6H8O6] at m/z 322, suggesting that HM3 might be a glucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of HM3, in which the aglycone ion fragment at m/z 322 was detected by ESI-MS. In comparison with the 1H NMR spectrum of berberine, the other proton signals (Table 1) of the glucuronic acid moiety were established from the NOESY spectrum. In this, the β-configuration of the glucuronic acid moiety was identified on the basis of the coupling constants of the anomeric proton (δ5.15, J = 7.0 Hz). Therefore, the structure of HM3 was identified as thalifendine-3-O-β-D-glucuronide.

HM4 (RM2) was obtained as a yellow amorphous powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of HM4 gave the quasi-molecular ion [M − Cl] at m/z 498. Bombardment of 498 afforded the fragment ion [M − Cl − C6H8O6] at m/z 322, suggesting that HM4 was a glucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of HM4. Comparison of the 1H NMR data (Table 1) of HM4 with those of HM3 indicated that HM4 was an isomer of HM3. The linked positions of the methoxyl group were established from the NOESY spectrum, in
URINARY METABOLITES OF BERBERINE IN RATS AND HUMANS

Fig. 1. ESI/MS spectra of [M – Cl]⁺ ion of berberine metabolites HM1 (A), HM2 (B), HM3 (C), HM5 (D), RM1 (E), and RM5 (F) in rat and human urine.
confirmed on the basis of the coupling constants of the anomeric and H-11 (NOESY spectrum, in which H-8 (8.01 (1H, d, 9.2 Hz) 8.11 (1H, d, 9.2 Hz) 8.04 (1H, d, 9.2 Hz) 7.89 (1H, d, 9.0 Hz) 8.04 (1H, d, 9.2 Hz) 8.19 (1H, d, 9.2 Hz) 5.14 (1H, d, 7.0 Hz) 5.15 (1H, d, 7.5 Hz) 4.82 (1H, d, 7.0 Hz) 4.85 (1H, d, 7.0 Hz) 2.3-OCH₃ 3.93 (3H, s) 3.95 (3H, s) 9-OCH₃ 4.10 (3H, s) 4.24 (3H, s) 4.09 (3H, s) 4.01 (3H, s) 4.05 (3H, s) GlcUA-1' 5.06 (1H, d, 7.5 Hz) 5.12 (1H, d, 6.0 Hz) 5.04 (1H, d, 7.0 Hz) s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapped; GlcUA, glucuronic acid.

**TABLE 2**

1H NMR data of berberine metabolites HM5-7, RM1, and RM5 in humans and rats

All spectra were recorded on an AV-600 spectrometer in DMSO-d₆ except that RM1 was dissolved in D₂O-DMSO-d₆ (1:2).

<table>
<thead>
<tr>
<th>No.</th>
<th>HM5</th>
<th>HM6 (RM4)</th>
<th>HM7</th>
<th>RM1</th>
<th>RM5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.73 (1H, s)</td>
<td>7.72 (1H, s)</td>
<td>8.09 (1H, s)</td>
<td>7.82 (1H, s)</td>
<td>7.52 (1H, s)</td>
</tr>
<tr>
<td>4</td>
<td>5.06 (d, 5.04 Hz)</td>
<td>5.04 (d, 5.04 Hz)</td>
<td>5.12 (d, 5.04 Hz)</td>
<td>5.12 (d, 5.04 Hz)</td>
<td>5.12 (d, 5.04 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>3.20 (2H, t, 6.2 Hz)</td>
<td>3.13 (2H, t, 6.0 Hz)</td>
<td>3.23 (2H, o)</td>
<td>3.15 (2H, o)</td>
<td>3.18 (2H, 1.6 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>4.95 (2H, t, 6.2 Hz)</td>
<td>4.88 (2H, t, 6.0 Hz)</td>
<td>4.96 (2H, m)</td>
<td>4.76 (2H, m)</td>
<td>4.89 (2H, 1.6 Hz)</td>
</tr>
<tr>
<td>8</td>
<td>9.91 (1H, s)</td>
<td>9.83 (1H, s)</td>
<td>9.87 (1H, s)</td>
<td>9.63 (1H, s)</td>
<td>9.71 (1H, s)</td>
</tr>
<tr>
<td>11</td>
<td>8.22 (1H, d, 9.2 Hz)</td>
<td>8.30 (1H, d, 9.0 Hz)</td>
<td>8.20 (1H, d, 9.0 Hz)</td>
<td>8.03 (1H, d, 9.0 Hz)</td>
<td>7.84 (1H, o)</td>
</tr>
<tr>
<td>12</td>
<td>8.05 (1H, d, 9.2 Hz)</td>
<td>7.84 (1H, d, 9.0 Hz)</td>
<td>8.02 (1H, d, 9.0 Hz)</td>
<td>7.99 (1H, d, 9.0 Hz)</td>
<td>7.84 (1H, o)</td>
</tr>
<tr>
<td>13</td>
<td>9.05 (1H, s)</td>
<td>8.93 (1H, s)</td>
<td>9.15 (1H, s)</td>
<td>8.72 (1H, s)</td>
<td>8.84 (1H, s)</td>
</tr>
</tbody>
</table>

s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapped; GlcUA, glucuronic acid.

which H-8 (δ10.15) exhibited correlations with H-6 (δ5.10, δ5.85) and H-11 (δ8.19) exhibited correlations with H-12 (δ5.06, δ5.04 Hz). Therefore, the methoxyl group was located at C-2 and C-3, respectively. The two methoxyl groups were identified as 1-O-b-D-glucuronide.

**HM7** was obtained as a yellow powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of **HM7** gave a quasi-molecular ion [M – Cl]⁺ at m/z 514. Bombardment of 514 afforded the fragment ion [M – Cl – C₆H₄O₆]⁺ at m/z 500, and consequent bombardment of 500 yielded [M – Cl – 2 × C₆H₄O₆]⁺ at m/z 324, suggesting that **HM7** might be a diglucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of **RM1**, in which the aglycone fragment at m/z 324 was detected by ESI-MS.

In comparison with the 1H NMR spectrum of berberine, most of the skeleton proton signals (Table 2) remained in RM1 except for loss of the characteristic methylene signal. The linked positions of the two glucuronic acid moieties and the two methoxyl groups were established by NOESY. The two anomic protons at δ8.12 (d, J = 7.0 Hz) and δ8.04 (d, J = 7.0 Hz) correlated with H-1 (δ7.82) and H-4 (δ8.05, d, J = 7.0 Hz), respectively, indicating that the two glucuronic acid moieties were located at C-2 and C-3, respectively. The two methoxyl groups at δ3.97(s) and δ3.92(s) correlated with H-11 (δ8.03, d, J = 9.0 Hz) and H-8 (δ6.93, s), respectively, suggesting that they should be linked to C-10 and C-9, respectively. The β-configurations of the two glucuronic acid moieties were confirmed from the coupling constants of the two anomic protons (δ8.12, J = 6.0 Hz; δ8.04, J = 7.0 Hz). Therefore, the structure of **RM1** was identified as columbamine-2-O-β-D-glucuronide.

**RM1** was obtained as a yellow powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of **RM1** gave a quasi-molecular ion [M – Cl]⁺ at m/z 676. Bombardment of 676 afforded the fragment ion [M – Cl – C₆H₄O₆]⁺ at m/z 500, and consequent bombardment of 500 yielded [M – Cl – 2 × C₆H₄O₆]⁺ at m/z 324, suggesting that **RM1** might be a diglucuronide conjugate. This suggestion was further confirmed by enzymatic hydrolysis of **RM1**, in which the aglycone fragment at m/z 324 was detected by ESI-MS.

In comparison with the 1H NMR spectrum of berberine, most of the skeleton proton signals (Table 2) remained in RM1 except for loss of the characteristic methylene signal. The linked positions of the two glucuronic acid moieties and the two methoxyl groups were established by NOESY. The two anomic protons at δ8.12 (d, J = 6.0 Hz) and δ8.04 (d, J = 7.0 Hz) correlated with H-1 (δ7.82) and H-4 (δ8.17), respectively, indicating that the two glucuronic acid moieties were located at C-2 and C-3, respectively. The two methoxyl groups at δ3.97(s) and δ3.92(s) correlated with H-11 (δ8.03, d, J = 9.0 Hz) and H-8 (δ6.93, s), respectively, suggesting that they should be linked to C-10 and C-9, respectively. The β-configurations of the two glucuronic acid moieties were confirmed from the coupling constants of the two anomic protons (δ8.12, J = 6.0 Hz; δ8.04, J = 7.0 Hz). Therefore, the structure of **RM1** was finally determined as demethylberberine-2,3-di-O-β-D-glucuronide.
FIG. 2. Structures of berberine metabolites in rat or human urine and possible metabolic pathways for their production.
Unconjugated Metabolite. RM5 was obtained as a red powder, giving a positive reaction to Dragendorff’s reagent. The ESI-MS of RM5 gave the quasi-molecular ion [M − Cl]⁺ at m/z 322, which is 14 mass units less than that of berberine. Comparison of the 1H NMR data (Table 2) with those of berberine showed there were many similarities except for the disappearance of one methyl group. The remaining methoxyl group was assigned to 9-O-Me on the basis of the NOESY spectrum, in which the methoxyl signal (δ 4.05) showed a correlation with H-8 (δ 7.71). After comparison of the 1H NMR data with those in the literature (Niu et al., 2006), RM5 was identified as thalifendine.

Discussion

Structural elucidation of metabolites is one of the most challenging tasks in drug metabolism studies. In recent years, comparisons of ESI-MSⁿ data and HPLC retention times with synthetic standards usually have been used to identify the structures of metabolites. However, when the standards are difficult to synthesize, some metabolite structures deduced only from LC/MSⁿ data may not be correct, especially if isomeric metabolites are present. In our study, three groups of isomers (HM1 and HM6, HM2 and HM7, and HM3 and HM4) were obtained, and they had identical LC/MSⁿ data. Therefore, their exact structures could not be identified from only LC/MSⁿ data (Zhu et al., 2007). In these cases, preparation of metabolites and further identification on the basis of NMR data are needed. Of course, direct isolation of the metabolites from urine, bile, or feces of humans or animals can be difficult, but it is the most reliable method for the identification of metabolites. We have determined the definitive structures of nine metabolites of berberine by enzymatic hydrolysis and examination of MS and NMR spectra. These results are important for a better understanding of its in vivo metabolic fate and disposition in rats and humans.

In this study, nine urinary metabolites of berberine in rats and humans were isolated and identified. On the basis of the metabolites, it is suggested that berberine is metabolized in both rats and humans mainly with the involvement of 2,3-OCH₂O and 9,10-OCH₃ by cleavage of the dioxymethylene five-membered ring or demethylation to form the corresponding phase I metabolites, some of which then underwent conjugation with glucuronic acid or sulfuric acid to form phase II metabolites. Most of the final metabolites were sulfate or glucuronide conjugates. These metabolites are very polar and are easily excreted.

To examine the possible site of metabolism, we analyzed the gastrointestinal contents of rats 4 h after oral administration of berberine and could not detect any metabolites, indicating that berberine is relatively stable in the gastrointestinal tract. However, we also investigated the urinary metabolites of berberine after intraperitoneal injection and obtained the same metabolites as those after oral administration, implying that the metabolites of berberine are formed after absorption rather than in gastrointestinal tract. On the basis of the structures of the metabolites isolated from the urine, possible metabolic pathways in rats and humans are proposed (Fig. 2).

Although berberine exhibits a variety of pharmacological effects in clinical situations, its poor oral bioavailability makes it hard for us to understand its clinical application for treatment of diabetes mellitus (Ni, 1988), hyperlipemia (Kong et al., 2004), arrhythmia, and heart failure (Zeng and Zeng, 1999; Lau et al., 2001; Zeng et al., 2003). This study demonstrated that the absorbed berberine is easily metabolized in vivo and its extensive biotransformation may be one of the main reasons for its low oral bioavailability.

Several reports have shown that a high concentration is necessary for berberine to display its in vitro bioactivities (Kong et al., 2004; Yin et al., 2008; Pyianuch et al., 2007). However, the blood-berberine concentration in animals or humans was very low when it was given orally in experimental or clinical doses (Shen et al., 1993; Yu et al., 2000), suggesting that the berberine metabolites might be responsible for the pharmacological effects. The bioactivities of the berberine metabolites including the corresponding phase I metabolites are now under investigation.

Acknowledgments.

We thank Dr. David Jack (United Kingdom) for the language check and the editorial assistance.

References


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