Identification of three sulfate-conjugated metabolites of berberine chloride in healthy volunteers' urine after oral administration

PAN Jun-Fang, YU Chen1, ZHU Da-Yuan2, ZHANG Hui1, ZENG Jia-Feng, JIANG Shan-Hao, REN Jian-Ying1 (State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031; 2Research Center, Shanghai Xu-hui District Central Hospital, Shanghai 200031, China)

KEY WORDS berberine; biotransformation; metabolism; biomolecular nuclear magnetic resonance; electrospray ionization mass spectrometry

ABSTRACT

AIM: To identify the structure of unknown metabolites of berberine (Ber) in human urine after oral administration. METHODS: Urine samples were obtained from 5 volunteers after they orally took Ber chloride 0.9 g per day for three days. Metabolites in urine samples were isolated and purified by polypropylene resin column chromatography. The individual metabolites were identified mainly using electrospray ionization mass spectrometry (ESI-MS) and proton nuclear magnetic resonance (1H NMR) spectroscopy. RESULTS: Three unknown metabolites (M1, M2, and M3) were isolated. They were susceptible to alylsulfatase. ESI-MS measurements of M1, M2, and M3 produced quasimolecular ions [M + H]⁺, m/z 417.9, 404.0, and 402.0 respectively. Especially, each of them produced a characteristic protonated ion [M·H⁺ + H]⁺, which can be ascribed as quasimolecular ions lost a SO₃ fragment. 1H NMR spectra of the metabolites were also obtained and each of 1H signals was assigned. CONCLUSION: Structures of M1, M2, and M3 were firmly identified as jatrorrhizine-3-sulfate, demethyleberberine-2-sulfate, and thalifendine-10-sulfate, and the major metabolite was M2.

INTRODUCTION

Berberine (Ber) is an isoquinoline quaternary ammonium alkaloid. It is an active constituent of Coptis rhizoma and many other plants which have been used in Chinese medicine for at least 3000 years. Ber chloride or sulfate, isolated from plants or chemically synthesized also has been clinically used as antibacterial, anti-diarrhoeal, and antiarrhythmic agent for more than thirty years.¹

There were several reports on the pharmacokinetics of Ber including its absorption, distribution, and excretion in animals²⁵ and man⁶. However, there was little information about the metabolic fate of Ber in animal or man.

While detecting the urinary excretion of Ber in men after oral administration, unknown metabolites were observed by a fluorescence-monitoring high performance liquid chromatography (HPLC) method.⁷ To know more about the metabolic fate of Ber in human body, study was carried out to separate and identify these metabolites in urine.

![Chemical structure of metabolites](image)

Berberine chloride: R₁, R₂ = OCH₂O, R₃ = OCH₃
Mₙ = 336 + 35.5

Demethyleberberine chloride: R₁ = R₂ = OH, R₃ = OCH₃
Mₙ = 324 + 35.5

Jatrorrhizine chloride: R₁ = R₂ = OCH₃, R₃ = OH
Mₙ = 338 + 35.5

Thalifendine chloride: R₁, R₂ = OCH₂O, R₃ = OH
Mₙ = 322 + 35.5

² Correspondence to Prof ZHU Da-Yuan, Phm 06-21-6431-1833, ext 318. E-mail dyzhu@mail.shcnc.ac.cn
Received 2001-05-14 Accepted 2001-10-14
MATERIALS AND METHODS

Drugs and reagents Ber chloride as sugar-coated tablet was provided by Shanghai Lisheng Pharmaceutical Co (Shanghai, China). Ber chloride and jatrorrhizine (Jat) chloride as chemical reagents were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Demethylenberberine (DMB) was synthesized from Ber in our laboratory. Porous resin 1400 was the product of Shanghai Institute of Pharmaceutical Industry (Shanghai, China). HSGP254 plates for thin layer chromatography (TLC) analysis were the products of Yantai Zhi Fu Huang Wu Silic Gel Factory (Yantai, China). Arylsulfatase (Type V, Product number S8629) was purchased from Sigma Chemical Co (San Jones, USA). Other chemicals were of commercially available reagent grade.

Instrument Finnigan MAT LCQ ion trap mass spectrometer coupled with electrospray ionization interface (Finnigan) and AM-400 spectrometer (Bruker) were used in electrospray ionization mass spectroscopy (ESI-MS) and proton nuclear magnetic resonance (1H NMR) measurement.

Urine samples collection Five healthy volunteers (male, 21 to 28 a) orally took Ber chloride tablets (100 mg per tablet) 900 mg per day for three days. Blank urine samples! were collected before the dosing. After starting the administration, 5-d urine were collected. All samples were stored at −20°C before the isolation procedure.

Isolation and purification Pooled urine samples (36 L) were applied to a glass column packed with 1 kg of porous resin 1400, which was previously activated with acetone and followed by 1% acetic acid. The column was then washed with 15 L 1% acetic acid. A stepwise elution gradient from 10% methanol in 1% acetic acid to 100% methanol was then applied. The eluate fraction was concentrated to a minor volume by a rotary evaporator under vacuum at 45°C. The blank urine sample was undergone the similar chromatography procedure.

Thin layer chromatography analysis TLC was performed with HSGP254 plates and developing solvents as n-butanol; acetic acid; water (4:1:1; v:v: v). Both 254 nm UV-light and the Dragendorff test were used as visualizing methods. The fractions containing metabolites were purified on Sephadex LH-20 columns with chloroform-methanol (1:1) solution as eluant solvent.

Enzymatic degradation Isolated metabolites were respectively incubated in 0.05 mol/L pH 7.4 Tris buffer solution with arylsulfatase 300 kU/L at 37°C for 15 h. The metabolites were detected by TLC before and after incubation.

Spectral analysis For ESI-MS measurement, isolated metabolites were separately dissolved in methanol and directly injected into the electrospray ionization interface of the mass spectrometer. The ionization was on a positive mode with a spray voltage of +4.25 kV. For 1H NMR measurements, metabolites were dissolved in Me3SO-d4, and the 1H NMR spectra were obtained at 400.14 MHz by an AM-400 spectrometer.

RESULTS

Three metabolites (M1, M2, and M3) were separated and detected in the fractions of 40% to 80% in methanol eluant by polyporous resin column chromatography. The amounts of M1, M2, and M3 thus purified were about 250, 17, and 2 mg, respectively. They all were yellow needle crystals.

The three metabolites were all degraded after they were incubated with arylsulfatase. This suggested they were sulfate conjugates. Furthermore, in TLC analysis, the product of M1 and M2 had the same Rf value and color reaction as the product of M2 as Jat chloride and DMB chloride, respectively.

Jat chloride and Ber chloride were used as referenced compounds for the ESI-MS test, and their spectra were shown in Fig 1. ESI-MS measurements of M1, M2, and M3 produced quasimolecular ions [M+H]+, m/z 417.9, 404.0, and 402.0, respectively. Especially, each of them produced a characteristic protonated ion [M-80+H]+, which can be ascribed as quasimolecular ions lost a SO4 fragment (Fig 2 and Fig 3). So the sulfate conjugate structure of these metabolites was confirmed. And the mass of three characteristic protonated ions [M-80+H]± were equal to that of Jat, DMB, and thalifendine (Tha), respectively.

To assign the conjugating position, 1H NMR data of M1, M2, and M3 were compared with that of Ber chloride and relative compounds (Tab 1). Proton signals of M1 were very similar to that of Jat chloride except that the H-4 was shifted 0.66 to lower field, which suggested there was an electron withdrawing group, namely -SO4 group, in C-3 position. Compared with DMB chloride, the H-1 of M2 was shifted 0.49 to
Fig 1. Electrospray ionization mass spectra of jatrorrhizine chloride (Jat, left) and berberine chloride (Ber, right).

Tab 1. $^1$H NMR data of M1, M2, M3, berberine chloride (Ber), demethyleberberine chloride (DMB), and jatrorrhizine (Jat) chloride in Me$_2$SO-d$_6$.

<table>
<thead>
<tr>
<th></th>
<th>OCH$_3$-2,3</th>
<th>OCH$_3$-2</th>
<th>H-4</th>
<th>2H-5</th>
<th>2H-6</th>
<th>H-8</th>
<th>OCH$_3$-9</th>
<th>OCH$_3$-10</th>
<th>H-11</th>
<th>H-12</th>
<th>H-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>7.73</td>
<td>3.92</td>
<td>7.56</td>
<td>3.20</td>
<td>4.94</td>
<td>9.91</td>
<td>4.10</td>
<td>4.07</td>
<td>8.22</td>
<td>8.04</td>
<td>9.05</td>
</tr>
<tr>
<td>s</td>
<td>8.02</td>
<td>6.92</td>
<td>3.18</td>
<td>4.92</td>
<td>9.86</td>
<td>4.08</td>
<td>4.06</td>
<td>8.19</td>
<td>8.13</td>
<td>8.81</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>7.83</td>
<td>6.17</td>
<td>7.08</td>
<td>3.18</td>
<td>4.91</td>
<td>9.85</td>
<td>4.26</td>
<td>8.32</td>
<td>7.85</td>
<td>8.89</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>7.80</td>
<td>6.17</td>
<td>7.09</td>
<td>3.20</td>
<td>4.98</td>
<td>9.90</td>
<td>4.09</td>
<td>4.07</td>
<td>8.21</td>
<td>8.06</td>
<td>8.94</td>
</tr>
<tr>
<td>Ber</td>
<td>7.83</td>
<td>6.17</td>
<td>7.09</td>
<td>3.20</td>
<td>4.98</td>
<td>9.90</td>
<td>4.09</td>
<td>4.07</td>
<td>8.21</td>
<td>8.06</td>
<td>8.94</td>
</tr>
<tr>
<td>DMB</td>
<td>7.53</td>
<td>6.86</td>
<td>3.10</td>
<td>4.89</td>
<td>9.83</td>
<td>4.07</td>
<td>4.04</td>
<td>8.16</td>
<td>8.04</td>
<td>8.74</td>
<td></td>
</tr>
<tr>
<td>Jat</td>
<td>7.71</td>
<td>3.95</td>
<td>6.90</td>
<td>3.15</td>
<td>4.93</td>
<td>9.87</td>
<td>4.10</td>
<td>4.08</td>
<td>8.20</td>
<td>8.03</td>
<td>9.03</td>
</tr>
</tbody>
</table>

Coupling pattern: d, doublet; t, triplet; s, singlet.
lower field, which indicated the conjugate position was at C-2. In $^1$H NMR spectrum of M3, the existence of only one methoxyl group was shown and H-11 was shifted 0.11 to the lower field while H-12 shifted 0.15 to the higher field compared with that of Ber chloride. On this observation it was considered that M3 was in vivo produced by demethylation of one of two methoxyl groups of Ber followed by sulfate conjugation at same position. The location of sulfate group at C-10 was determined by nuclear overhauser effect (NOE) experiments; enhancement of a single aromatic signal (H-8) by 5.3 % was observed when the -OCH$_3$ was irradiated.

On the findings above, M1, M2, and M3 were jatrorrhizine-3-sulfate, demethylenberberine-2-sulfate, and thalifendine-10-sulfate (Fig 3).

DISCUSSION

The polypropylene resin 1400 is a kind of polystyrene packing material, which is usually used as solid extracting material in hydrophilic natural product research. In the present study the resin was used as chromatographic material, and three high-polar metabolites of Ber were successfully isolated from human urine.

Several techniques, including enzyme hydrolysis together with acid hydrolysis and ESI/MS experiments, provided strong evidence for the sulfate conjugate structure of these unknown metabolites. The susceptibility to arylsulfatase and mineral acid is in accordance with the behavior of other sulfate conjugates$^{[8]}$. However it was particular in ESI/MS that produced direct proof of the conjugating group. This novel MS technique, which is aimed at structure identification of highly polar and labile molecules, not only provided the molecular weight of the sulfate ester but also, through the intense fragments from the molecule ion, clearly established the conjugating group as sulfate$^{[9]}$. However, the $^1$H NMR data of every purified metabolite was necessary for the final
metabolite be found in urine. DMB, Jar, and Tha as possible phase I metabolite may have relatively higher concentrations and not very short retention periods in blood. For these compounds had shown varied bioactivities as that of Ber, they should not be ignored when looking into the system effect of Ber.

REFERENCES


Fig 3. Structures and MS fragmentation of three metabolites (M1, M2, and M3) of berberine (ber) in human urine.

In the present study, little Ber was recovered in urine as compared with its metabolites. This result was in accordance with our early discovery that only a total urinary excretion of 27.49 μg was detected after oral administration of 0.2 g Ber chloride in men. Among the three isolated metabolites, M2, namely demethylsarpentine-2-sulfate, had an absolutely higher amount. This result means that demethyl reaction following sulfate conjugation is a major metabolite route of berberine in man.

Although, in present study there was no phase I...
关键词 小檗碱；生物转化；代谢；活质分子核磁共振；电子喷雾离子化质量光谱法

目的：分离和鉴定健康志愿者口服盐酸小檗碱后尿中的未知代谢产物。方法：用大孔树脂柱层析方法分离和纯化代谢产物，电喷雾-离子化质谱（ESI-MS）及1H核磁共振（1H NMR）进行结构分析。结果：三个未知代谢产物（M1、M2和M3）被分离纯化，它们均对芳基磺酸酶敏感。电喷雾质谱中分别可见准分子离子峰[M+H]+417.9，404.0和402.0，以及脱SO3片段的特征离子峰[M-80+H]+。测得各自的1H NMR谱，参考盐酸小檗碱的质子信号，确定了M1、M2和M3的质子信号归属及取代基的位置。结论：M1、M2和M3的结构可分别推定为药用碱-3-硫酸酯（M1）、脱亚甲基小檗碱-2-硫酸酯（M2）和Thalifendine-10-硫酸酯（M3）。其中M2为主要代谢产物。

（责任编辑 未倩容）

本刊加人“万方数据——数字化期刊群”的声明

为了实现科技期刊编辑、出版发行工作的电子化，推进科技信息交流的网络化进程，我刊现已入网“万方数据——数字化期刊群”，所以，向本刊投稿并录用的稿件文章，将一律由编辑部统一收入“万方数据——数字化期刊群”，进入因特网信息服务。凡有不同意者，请另投它刊。本刊所付稿酬包含刊物内容上网服务报酬，不再另付。

“万方数据——数字化期刊群”是国家“九五”重点科技攻关项目。本刊全文内容按照统一格式制作，读者可上网查询浏览本刊内容，并征订本刊。