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

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

ABSTRACT

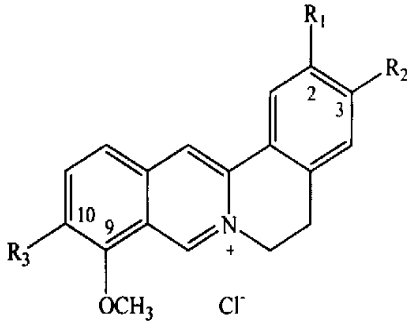
AIM: To identify the structure of unknown metabolites of berberine (Ber) in human urine after oral administration. **METHODS**: Urine samples were obtained from 5 volunteers after they orally took Ber chloride 0.9 g per day for three days. Metabolites in urine samples were isolated and purified by polyporous resin column chromatography. The individual metabolites were identified mainly using electrospray ionization mass spectroscopy (ESI-MS) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. **RESULTS**: Three unknown metabolites (M1, M2, and M3) were isolated. They were susceptible to arylsufatase. 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 SO_3 fragment. ^{1}H NMR spectra of the metabolites were also obtained and each of ^{1}H signals was assigned. **CONCLUSION**: Structures of M1, M2, and M3 were firmly identified as jatrorrhizine-3-sulfate, demethyleneberberine-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, antidiarrheal, 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 animals[2-5] and man[6]. 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.



Berberine chloride: $R_1, R_2 = OCH_2O$, $R_3 = OCH_3$
$M_r = 336 + 35.5$
Demethyleneberberine chloride: $R_1 = R_2 = OH$, $R_3 = OCH_3$
$M_r = 324 + 35.5$
Jatrorrhizine chloride: $R_1 = R_3 = OCH_3$, $R_2 = OH$
$M_r = 338 + 35.5$
Thalifendine chloride: $R_1, R_2 = OCH_2O$, $R_3 = OH$
$M_r = 322 + 35.5$

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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). Demethyleneberberine (DMB) was synthesized from Ber in our laboratory. Porous resin 1400 was the product of Shanghai Institute of Pharmaceutical Industry (Shanghai, China). HSGF254 plates for thin layer chromatography (TLC) analysis were the products of Yantai Zhi Fu Huang Wu Silic Gel Factory (Yantai, China). Arylsufatase (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 (^{1}H 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 ℃ 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 ℃. The blank urine sample was undergone the similar chromatography procedure.

Thin layer chromatography analysis TLC was performed with HSGF254 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 ℃ 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 ^{1}H NMR measurements, metabolites were dissolved in Me_2SO-d_6, and the ^{1}H 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 R_f 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 SO_3 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, ^{1}H 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 -SO_4 group, in C-3 position. Compared with DMB chloride, the H-1 of M2 was shifted 0.49 to

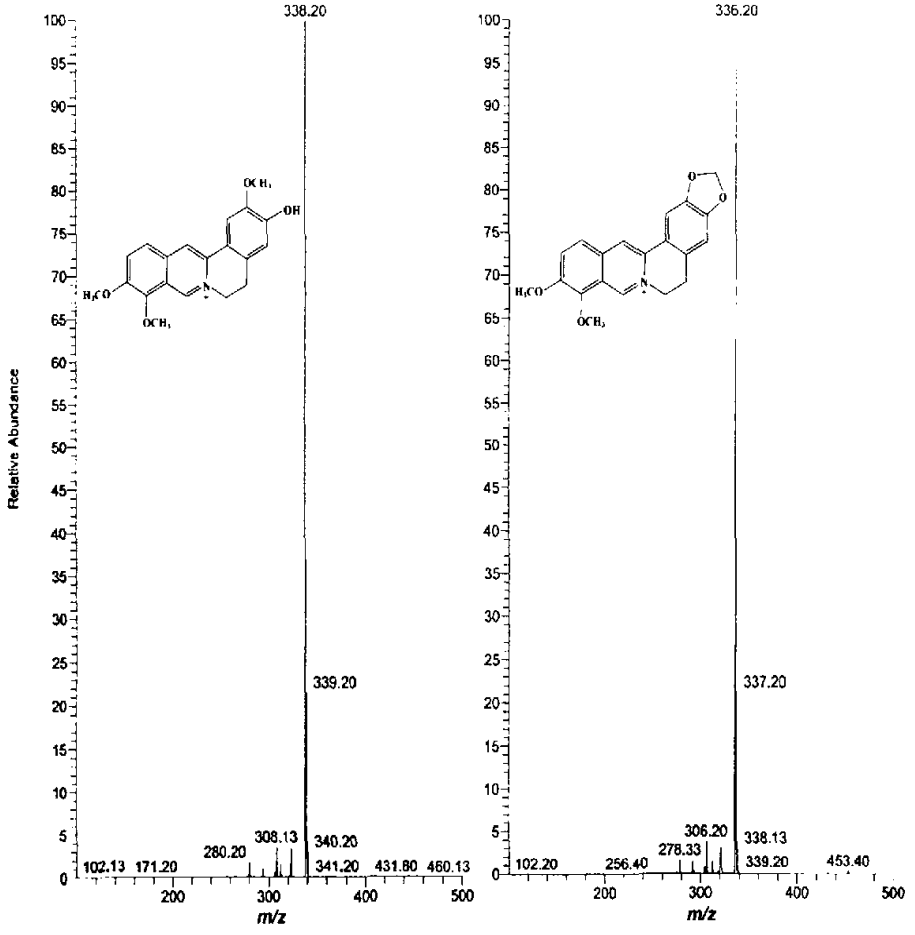

Fig 1. Electrospray ionization mass spectra of jatrorrhizine chloride (Jat, left) and berberine chloride (Ber, right).

Tab 1. ^{1}H NMR data of M1, M2, M3, berberine chloride (Ber), demethyleneberberine chloride (DMB), and jatrorrhizine (Jat) chloride in Me_2SO-d_6.

	H-1	OCH_2O -2,3	OCH_3-2	H-4	2H-5	2H-6	H-8	OCH_3-9	OCH_3-10	H-11	H-12	H-13
M1	7.73	–	3.92	7.56	3.20	4.94	9.91	4.10	4.07	8.22	8.04	9.05
	s	–	s	s	t	t	s	s	s	d	d	s
M2	8.02	–	–	6.92	3.18	4.92	9.86	4.08	4.06	8.19	8.13	8.81
	s	–	–	s	t	t	s	s	s	d	d	s
M3	7.83	6.17	–	7.08	3.18	4.91	9.85	4.26	–	8.32	7.85	8.89
	s	s	–	s	t	t	s	s	–	d	d	s
Ber	7.80	6.17	–	7.09	3.20	4.93	9.90	4.09	4.07	8.21	8.00	8.94
	s	s	–	s	t	t	s	s	s	d	d	s
DMB	7.53	–	–	6.86	3.10	4.89	9.83	4.07	4.04	8.16	8.04	8.74
	s	–	–	s	t	t	s	s	s	d	d	s
Jat	7.71	–	3.95	6.90	3.15	4.93	9.87	4.10	4.08	8.20	8.03	9.03
	s	–	s	s	t	t	s	s	s	d	d	s

Coupling pattern: d, doublet; t, triplet; s, singlet.

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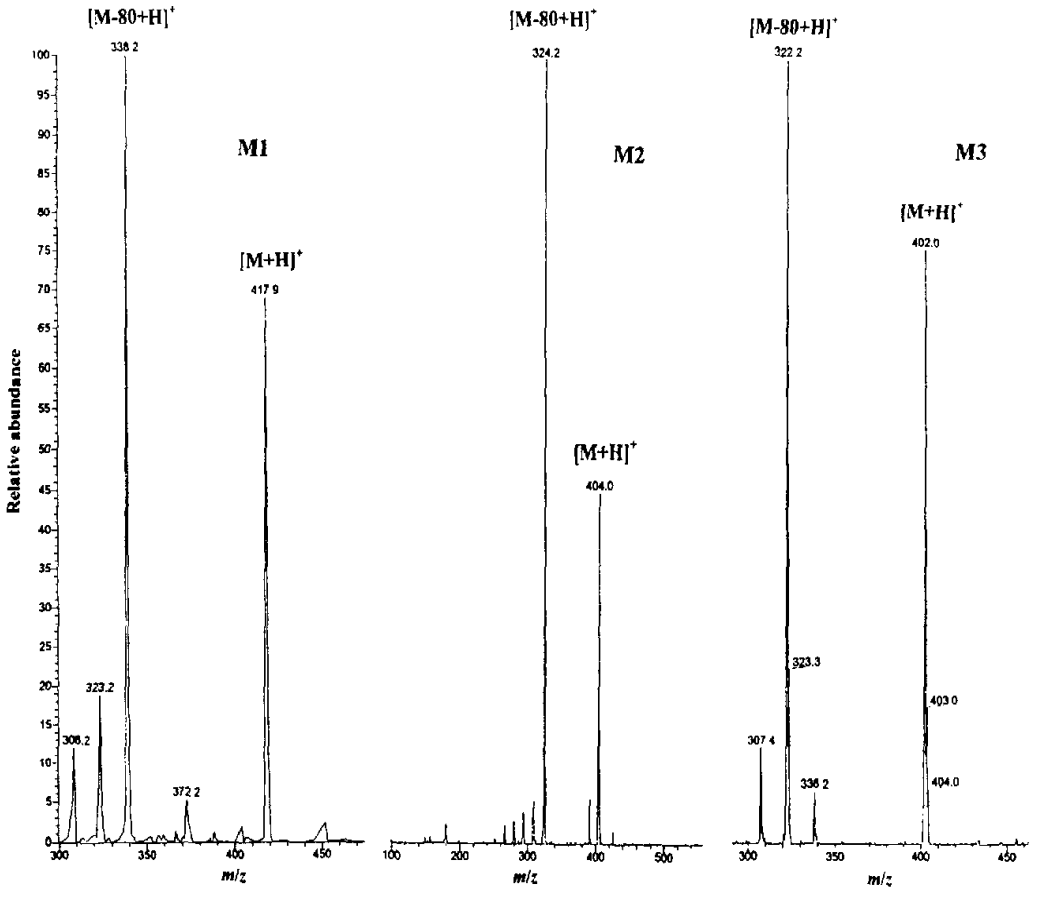


Fig 2. Electrospray ionization mass spectra of M1, M2, and M3.

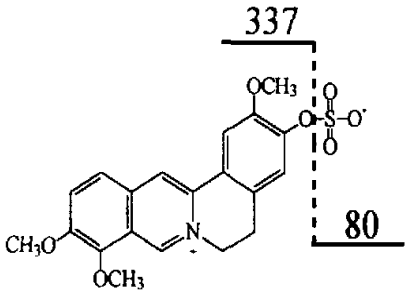
lower field, which indicated the conjugate position was at C-2. In ^{1}H NMR spectrum of M3, the existence of only one methoxyl group was shown and H-11 was shifted 0.11 to the lower field while H-12 shifted 0.15 to the higher field compared with that of Ber chloride. On this observation it was considered 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, demethyleneberberine-2-sulfate, and thalifendine-10-sulfate (Fig 3).

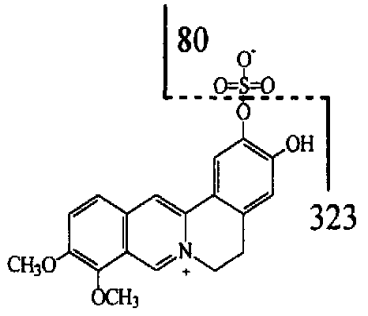
DISCUSSION

The polyporous 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.

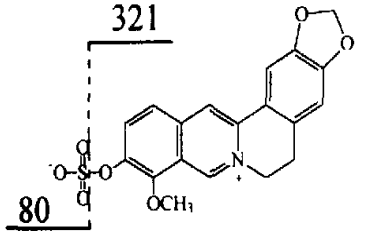
Several techniques, including enzyme hydrolysis together with acid hydrolysis and ESI/MS experiments, provided strong evidence for the sulfate conjugate structure of these unknown metabolites. The susceptibility to arylsulfatase and mineral acid is in accordance with the behavior of other sulfate conjugates[8]. 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



Jatrorrhizine-3-sulfate (M1)



Demethyleneberberin-2-sulfate (M2)



Thalifendine-10-sulfate (M3)

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

structure identification.

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 urinal excretion of 27.49 μg was detected after oral administration of 0.2 g Ber chloride in men[7]. Among the three isolated metabolites, M2, namely demethyleneberberine-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 metabolite be found in urine, DMB, Jat, and Tha as possible phase Ⅰ metabolite may had relatively higher concentrations and not very short retention periods in blood. For these compounds had shown varied bioactivities as that of Ber[10,11], they should not be ignored when looking into the system effect of Ber.

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健康志愿者口服盐酸小檗碱后尿中三个硫酸结合型代谢产物的鉴定

R96 A

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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**: 用大孔树酯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^{1}H核磁共振(^{1}H 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, 它们均对芳基硫酸酯酶敏感. 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SO_3片段的特征离子峰$[M-80+H]^+$. 测得各自的^{1}H NMR 谱, 参考盐酸小檗碱的质子信号, 确定了 M1, M2 和 M3 的质子信号归所及取代基的位置. **结论**: M1, M2 和 M3 的结构可分别推定为药根碱-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.

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