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Characterization of new metabolites from in vivo biotransformation of norisoboldine by liquid chromatography/mass spectrometry and NMR spectroscopy

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ABSTRACT

Norisoboldine (1,9-dihydroxy-2,10-dimethoxynoraporphine) is one of the major bioactive isoquinoline alkaloids in Linderae Radix, a commonly used Chinese herbal medicine. The aim of this study is to isolate and characterize metabolites of norisoboldine after gavage feeding in rats. High-performance liquid chromatography coupled with electrospray ionization and ion-trap mass spectrometry (HPLC-ESI/MS^n) was used to identify metabolites of norisoboldine in rat urine and bile samples. A total of five metabolites of norisoboldine were characterized by comparing retention time and UV absorption in HPLC, and by molecular mass and fragmentation pattern of the analytes by mass spectrometry with those of norisoboldine. Two new glucuronide conjugates of norisoboldine, noriosboldine-1-*O*-β-D-glucuronide and norisoboldine-9-*O*-α-D-glucuronide, were isolated from rat urine samples and their structures were confirmed by NMR spectroscopy (^{1}H, ^{13}C, HMBC and HSQC) for the first time. The results suggested that glucuronidation and sulfation were involved in metabolic pathways of norisoboldine in rat.

1. Introduction

Linderae Radix, dry roots of *Lindera aggregata* (Sims) Kosterm., a well-known traditional Chinese herbal medicine, is commonly used to treat several symptoms such as chest and abdominal pain, indigestion, regurgitation, cold hernia and frequent urination [1]. Phytochemical investigations on Linderae Radix have resulted in the discovery of several groups of bioactive components including isoquinoline alkaloids [2,3], sesquiterpene lactones [4,5], flavonoids [6] and tannins [7]. The total alkaloids extract from Linderae Radix can effectively reduce inflammation and joint destruction in type II collagen-induced arthritis, an animal model of human rheumatoid arthritis (RA). A series of isoquinoline alkaloids from the total alkaloids extract such as norisoboldine, linderaline, pallidine, protosinomenine, boldine and laurolitsine were obtained in our laboratory. Norisoboldine (Fig. 1) was identified as the most representative bioactive component of the total alkaloids extract [5,8,9], and hence the compound was regarded as a potential and promising lead drug for treating rheumatoid arthritis. However, until now no publication on the *in vivo* metabolism of norisoboldine has been reported. Hence *in vivo* study on the metabolism of norisoboldine will provide important information on the pharmacology of this compound. The results will also play a crucial role in the development and clinical application of this potential new drug.

Structure characterization of metabolites is a key to drug metabolism studies. Recently, high-performance liquid chromatography coupled to mass spectrometry has become a powerful technique for isolating and identifying structures of unknown metabolites present in biological samples due to its high sensitivity and specificity [10–12]. On this basis, HPLC-UV/MS^n was applied to analyze the metabolites of norisoboldine in rat urine and bile samples after gavage feeding of 40 mg/kg norisoboldine for the first time. In this study we aimed to characterize metabolites of norisoboldine and to establish possible metabolic pathways in rat.

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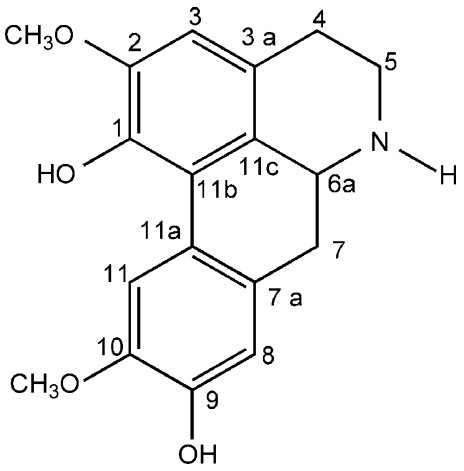

Fig. 1. Structure of norisoboldine.

2. Experimental

2.1. Materials, chemicals and reagents

Norisoboldine (with a purity of 99.0%) was obtained from Shanghai R&D Centre for Standardization of Chinese Medicines, Shanghai, China. HPLC-grade acetonitrile and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water (>18 mΩ) was obtained from a Milli-Q water purification system (Bedford, MA, USA). HPLC-grade formic acid was obtained from Tedia (Fairfield, OH, USA). Other chemicals used were analytical grade.

Reverse-phase silica gel (C_{18}) and MCI Gel CHP 20P (75–150 μm) for column chromatography were purchased from Merck (Darmstadt, Germany) and Mitsubishi (Tokyo, Japan), respectively.

2.2. Animals and drug administration

Male Sprague–Dawley rats weighting 250–300 g were provided by the Experimental Animal Centre, Shanghai University of Traditional Chinese Medicine, China. Animals were kept in an

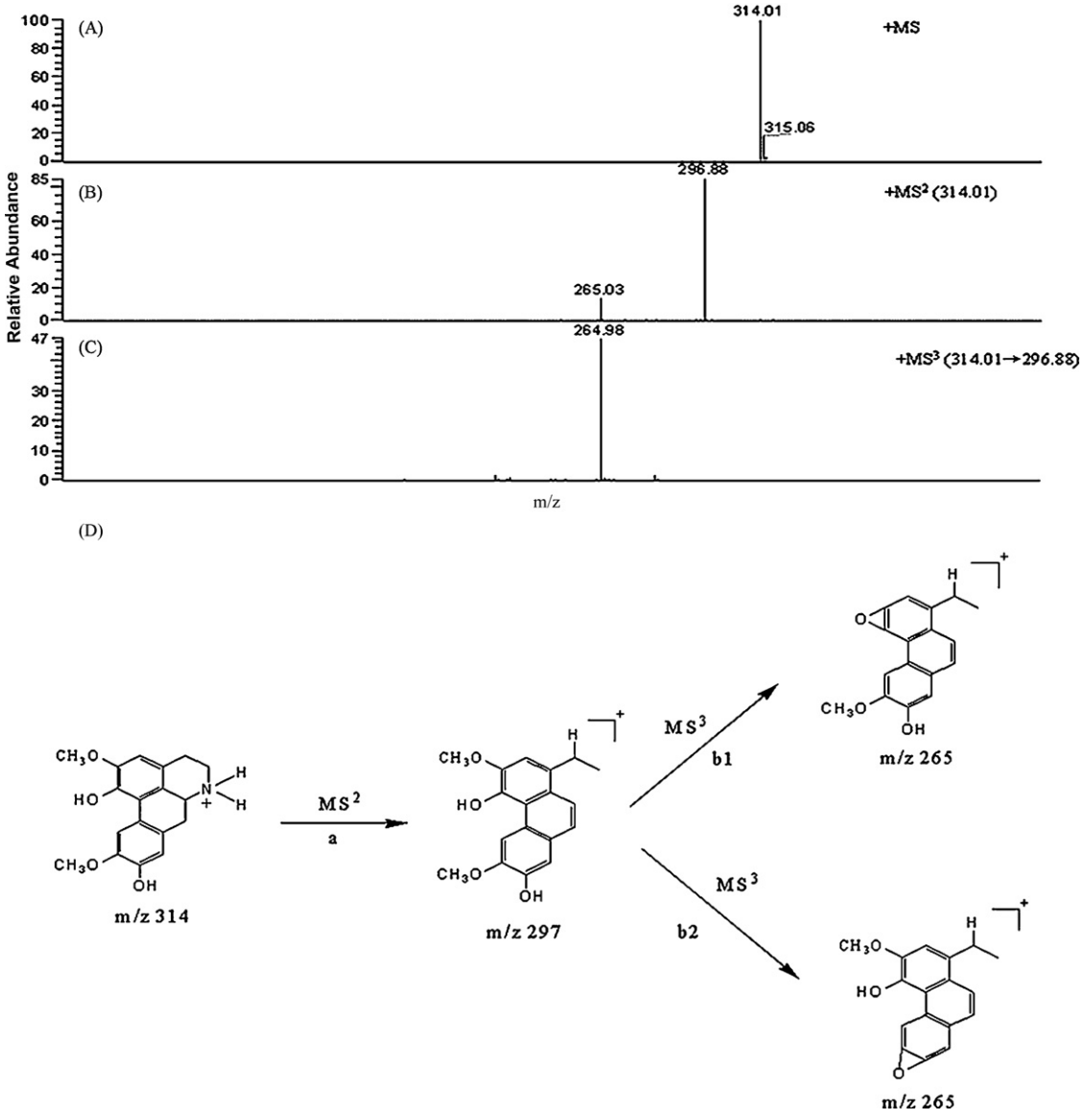

Fig. 2. (A) MS spectra of norisoboldine; (B) MS^2 spectra of norisoboldine; (C) MS^3 spectra of norisoboldine; (D) The proposed fragmentations pathways of norisoboldine.

environmentally controlled breeding room for 7 days before commencing the experiments. They were fed with standard laboratory food and water *ad libitum* and fasted for 12 h but with access to water prior to the administration of norisoboldine. All studies on animals were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China. Norisoboldine was dissolved in deionized water (8 mg/ml) and administered by gavage at a dose of 40 mg/kg body weight.

2.3. Samples collection and processing procedures

For urine sampling, six rats were housed in separate metabolic cages with free access to water. Urine samples were collected from 0 to 24 h after oral administration of norisoboldine. For bile sampling, six rats were fixed on a wooden plate and anesthetized with 20% urethane (7.0 ml/kg) through the abdomen. An abdominal incision was made and the common bile duct was cannulated with a Closed IV Catheter System (ID = 0.07 cm, Becton Dickinson, USA) for the collection of bile samples. Bile samples were collected from 0 to 6 h after oral administration of norisoboldine. Baseline rat urine and bile were collected before oral administration of norisoboldine. All samples were stored at −20 °C until analysis.

Each sample of urine (1 ml) and bile (1 ml) was mixed with 1 ml of 50% methanol, thoroughly, and centrifuged at 2432 × *g* for 10 min. The supernatant (1 ml) of each sample was collected and evaporated to dryness under nitrogen at 40 °C. The residue of each sample was re-constituted in 200 μl of 50% methanol. After centrifuging at 20,000 × *g* for 10 min, a 5 μl aliquot of the supernatant of an individual sample was injected into the chromatographic systems for identification of norisoboldine and its metabolites.

2.4. Isolation and purification of two urinary metabolites (M1 and M2)

In order to ascertain the structure of metabolites by NMR, larger quantities of metabolites were needed. Twenty normal rats were housed and administrated by gavage at a dose of 100 mg/kg, two times per day for 20 days. A total of 4 L of urine was collected and stored at −20 °C. Urine samples were subjected to MCI Gel CHP 20P chromatography (4.5 × 60 cm; 75–150 μm, 500 ml) and eluted with a MeOH–H_2O solvent system (0:100; 10:90; 30:70; 3.5 L, each). The 30% MeOH fractions (100 ml, each) were concentrated to almost dryness in vacuo after collection. The residue was dissolved in water. The solution was then subjected to a RP-18 silica gel chromatography (2.5 × 60 cm, 70 g) with a MeOH–H_2O solvent system (10:90). The fractions (10 ml, each) containing the metabolites were further purified by semi-preparative HPLC. The metabolites in each fraction were detected using HPLC-IT/MS mentioned in Sections 2.5.1 and 2.5.2.

Semi-preparative HPLC was performed with an ODS column (Kromasil 100 C_{18}, 250 × 10.0 mm, 5 μm, EKA Chemicals, Sweden) in a Waters 600 liquid chromatography apparatus equipped with a Waters 2996 PDA detector (Waters, Milford, MA). The detection wavelength was set at 280 nm. Elution with acetonitrile–0.1% formic acid (13:87, v/v) yielded 5 mg of *M1* (R.t. = 7.5 min) and 10 mg of *M2* (R.t. = 9.9 min) at a flow rate of 3 ml/min.

2.5. Instrumentation

2.5.1. Liquid chromatography

The analyses were performed with a Surveyor HPLC system (Thermo-Finnigan, San Jose, CA, USA) equipped with a quaternary gradient pump for mass spectrometry, an autosampler and a PDA detector. The components were separated on a Capcell PAK C_{18} reversed phase column (75 × 2.0 mm, 3 μm, Shiseido, Japan), which was coupled to a Capcell PAK C_{18} guard column (Shiseido, Japan). The components were eluted with a gradient system consisting of (A) acetonitrile and (B) 0.1% formic acid. The linear gradient program was used as follows (T min/% B): 0/95, 5/95, 12/80, 18/50, 25/5, 30/5, 31/95, 35/95. The PDA detector wavelength was set in the range of 200–800 nm. The mobile phase flow rate was 0.2 ml/min and column temperature was maintained at 25 °C.

2.5.2. Ion-trap mass spectrometry

Mass spectrometry was performed using a Finnigan LCQ DECA XP plus ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) equipped with an electrospray ion (ESI) source operating in positive ion mode. The optimized parameters were as follows: electrospray needle voltage, 5 kV; sheath gas flow, 32 arbitrary unit; auxiliary nitrogen gas flow, 10 arbitrary unit; capillary voltage, 21 V; heated capillary temperature of 300 °C. The relative collision energy for CID was adjusted at 40% of the maximum to acquire satisfactory product ion spectra. Data acquisition was performed in full-scan, selective ions monitoring (SIM) and MS^n modes in the range of *m*/*z* 100–1000. All operations were controlled by Xcalibur software version 1.2 (Finnigan).

2.5.3. NMR spectroscopy

A Bruker AV 500 NMR spectrometer (Faellanden, Switzerland) was used to record ^1H NMR (500 MHz), ^{13}C NMR (125 MHz), HMBC and HSQC spectra in D_2O at 27 °C, and chemical shifts are presented in ppm referenced to 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt.

2.5.4. High resolution mass spectrometry

High resolution mass spectrometry (HRMS) were recorded using a Waters Micromass hybrid quadrupole time-of-flight (Q-TOF™) mass spectrometer (Waters Technologies, Milford, USA) equipped with an ESI source and operated in positive ion mode with the mass scan range of *m*/*z* 100–800. The tuning parameters were optimized and set as follows: capillary voltage, 2 kV; sampling cone voltage, 30 V; extraction cone voltage, 1 V; source temperature, 100 °C; desolvation temperature, 350 °C; cone gas flow, 50.0 L/h; desolvation gas flow, 500.0 L/h. Data processing was performed on MassLynx 4.1 software package.

3. Results and discussion

3.1. Mass spectral analysis of norisoboldine

The MS^n fragmentation pattern of norisoboldine was examined to aid a better understanding of the MS^n spectra of its metabolites. The protonated molecular ion showed a predominant ion at *m*/*z* 314 (Fig. 2A). The MS^2 spectra of the ion at *m*/*z* 314 displayed two major product ions at *m*/*z* 297 and 265 (Fig. 2B). The MS^2 product ion at *m*/*z* 297 led to a MS^3 product ion at *m*/*z* 265 (Fig. 2C). The ion at *m*/*z* 297 was probably formed by the loss of the amino group as indicated in pathway a (Fig. 2D). The other ion at *m*/*z* 265 was formed by the loss of a molecule of methanol from the $[M+H-NH_3]^+$, as indicated in pathway b1 or b2 in Fig. 2D [13]. The detection of these characteristic product ions has suggested these are the metabolites of norisoboldine *in vivo*.

3.2. Identification of norisoboldine metabolites

Careful analysis of the data collected from the HPLC-IT/MS system resulted in the discovery of five metabolites in rat bile (*M1*–*M5*) and three metabolites in rat urine (*M1*, *M2* and *M4*). Their $[M+H]^+$ ions were at *m*/*z* 490, 490, 570, 394, 474 for *M1*, *M2*, *M3*, *M4* and *M5*, respectively. The MS^n spectra of these metabolites are shown

in Fig. 3. The λ_{max} observed in the UV spectra of these five metabolites were at 235, 285 and 300 nm, suggesting the metabolites are similar to the chromophore framework as in norisoboldine. All metabolites were identified by comparing UV spectra, the changes in observed mass ($\Delta m/z$) and MS^n spectra of metabolites with those of norisoboldine. The structures of *M1* and *M2* were further elucidated by NMR spectroscopy. All metabolites were glucuronide and/or sulfate conjugates, and the linkage positions were all not situated at the nitrogen atom but at the 1- or 9-hydroxyl position.

3.2.1. Metabolite M0

The LC retention time, UV absorption (λ_{max}), MS and MS^2 spectra of the protonated molecular ion at m/z 314 were the same as those of the norisoboldine standard. Therefore, it is identified as the non-metabolized norisoboldine (*M0*).

3.2.2. Metabolites M1 and M2

Metabolites *M1* and *M2* were observed at retention times of 10.59 and 11.65 min, respectively, with the same protonated molecular ion at m/z 490 (m/z 314 + 176 Da). The HRMS showed the

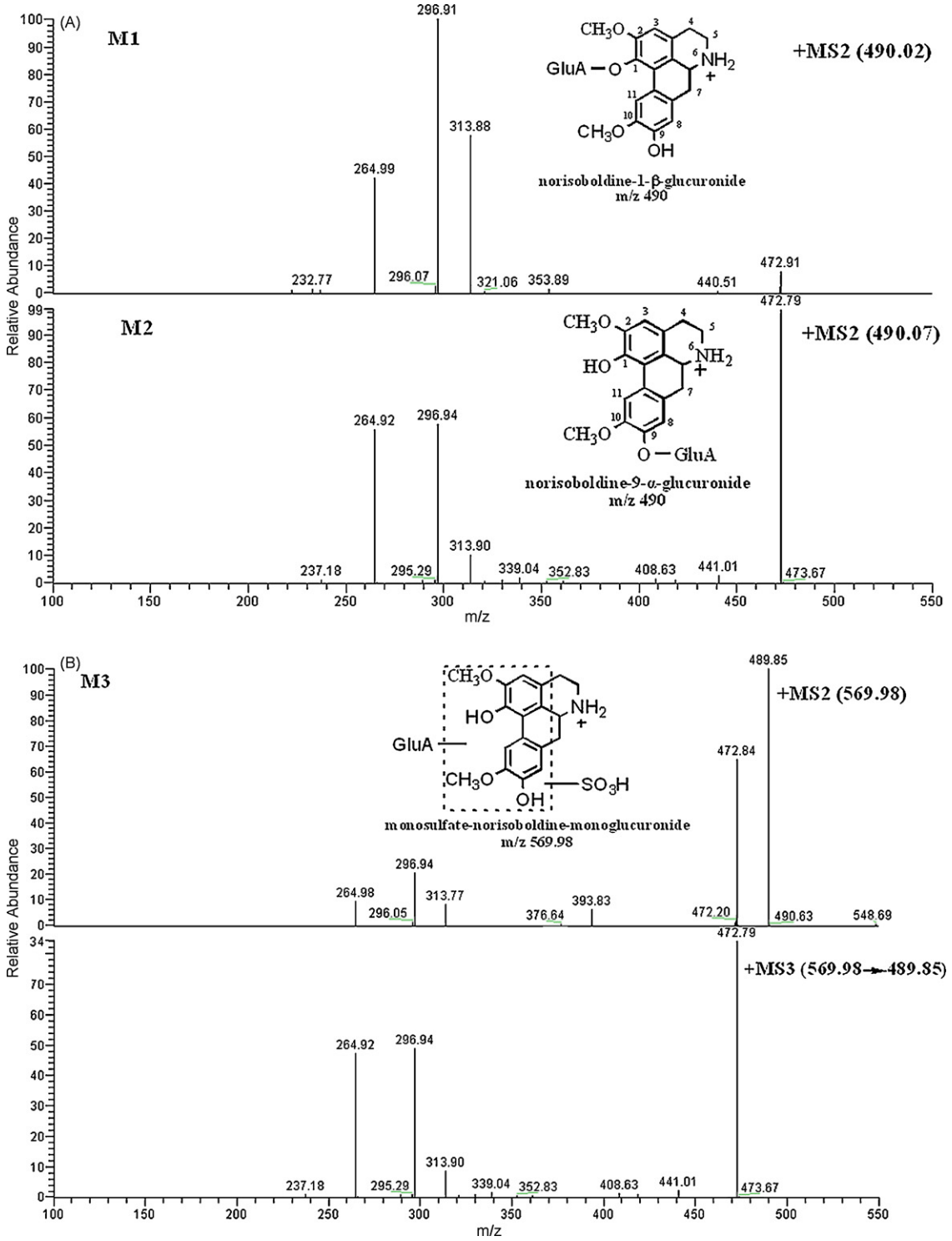

Fig. 3. MS^n spectra of metabolites (*M1–M5*) of norisoboldine using ESI in positive ion mode.

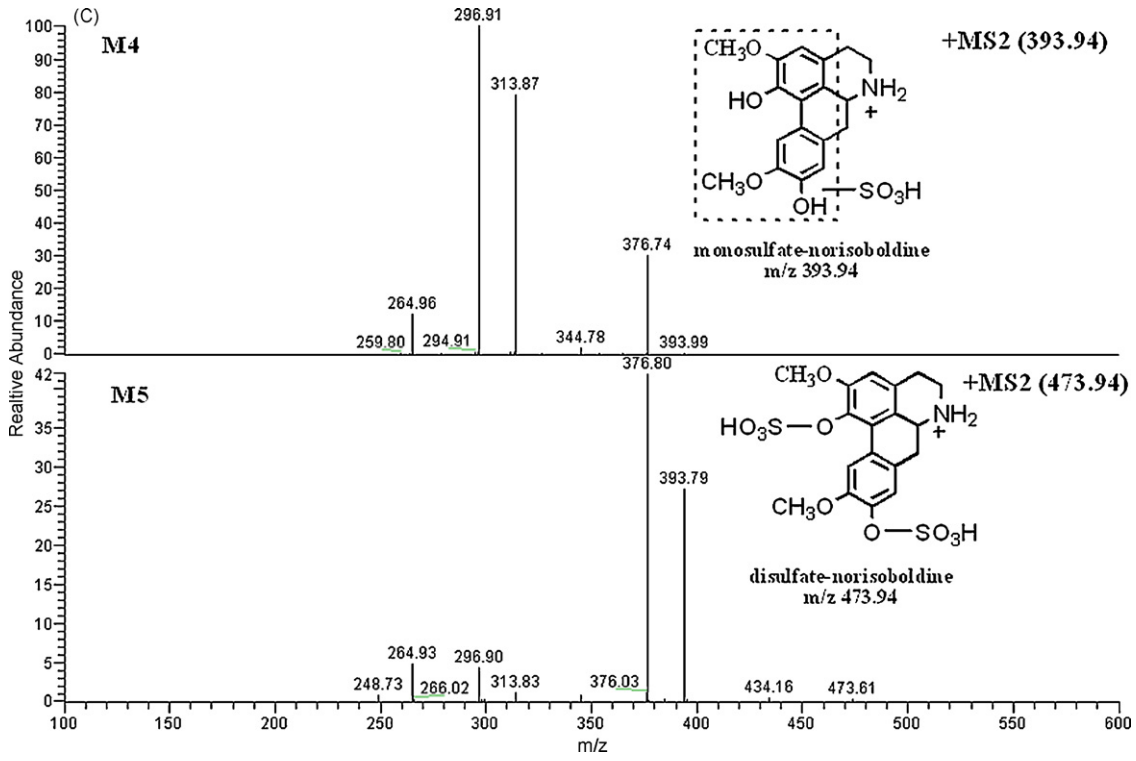

Fig. 3. (Continued)

protonated molecular ion at *m/z* 490.1558 (calculated 490.1713) for *M1* and 490.1597 (calculated 490.1713) for *M2*, corresponding to the same molecular formula $C_{24}H_{28}NO_{10}$. The molecular formulas of these two metabolites were further supported by the ^{1}H NMR and ^{13}C NMR spectral data. The MS^2 spectra of *M1* and *M2* displayed major product ions at *m/z* 265, 297, 314 and 473 (Fig. 3A). The protonated molecular ion of them and their main MS^2 product ion at *m/z* 473 were 176 Da (a glucuronic acid moiety) more than that of norisoboldine and its main MS^2 product ion (*m/z* 297). The characteristic MS^2 product ions and $[M+H]^+$ ion of norisoboldine at *m/z* 265, 297 and 314 were also present in the MS^2 spectra of *M1* and *M2*. These results indicated that the two metabolites were isomers of glucuronide conjugates of norisoboldine. Furthermore, the presence of the product ion, *m/z* 473, formed by the loss of the amino group from *m/z* 490 strongly suggested that the glucuronidated position was located at 1- or 9-hydroxyl group.

At the same time the ^{13}C NMR data of *M1* (Table 1) also clearly showed the existence of a glucuronic acid unit [δ 102.6 (C-1′), 75.5 (C-2′), 79.4 (C-3′), 74.4 (C-4′), 78.0 (C-5′), 176.9 (C-6′)] [14]. The ^{13}C NMR data of the aglycone part of *M1* (Table 1) were similar to those of norisoboldine (Table 1) [2], except for the following: the carbon signals of C-1 (δ 152.6), C-2 (δ 147.1) and C-3a (δ 125.1) were shifted downfield by 0.7, 1.6 and 2.9 ppm, respectively. The HMBC spectra showed cross peaks between C-1 (δ 152.6) and H-1′ (δ 5.23). Thus, *M1* was characterized as noriosboldine-1-*O*-β-D-glucuronide. The β-anomeric configuration of the glucuronic acid was determined from its coupling constant of the anomeric proton (J = 6.0 Hz) [15].

The ^{13}C NMR data of *M2* (Table 1) also clearly showed the existence of a glucuronic acid moiety [δ 99.4 (C-1′), 75.3 (C-2′), 79.0 (C-3′), 74.7 (C-4′), 78.0 (C-5′), 178.5 (C-6′)]. The ^{13}C NMR data of the aglycone part of *M2* were similar to those of norisoboldine, except for the following: the carbon signals of C-9 (δ 145.8) and C-8 (δ 114.7) were shifted upfield by 1.4 and 2.3 ppm, respectively, whereas the carbon signal of C-11a was shifted downfield by 2 ppm. The HMBC spectra showed cross peaks between C-9 (δ 145.8) and H-1′ (δ 5.36). Thus, the glucuronic acid was assigned to the C-9 position of norisoboldine. The α-anomeric configuration for glucuronic acid was determined from its small $^3J_{H1',\ H2'}$ coupling constant (J = 4.0 Hz) [15]. Hence *M2* was identified as norisoboldine-9-*O*-α-D-glucuronide.

3.2.3. Metabolite M3

The metabolite *M3* was found in rat bile sample only. The protonated molecular ion of *M3* was observed at *m/z* 570 (*m/z* 314 + 176 + 80 Da) with a retention time of 11.64 min. Its MS^2 spectra showed major product ions at *m/z* 265, 297, 314, 473 and 490. The MS^2 product ion at *m/z* 490 showed major MS^3 product ions at *m/z* 265, 297, 314 and 473. The protonated molecular ion of *M3* was 80 Da (equivalent to a sulfate unit) more than that of *M1* and *M2*. The MS^2 product ions at *m/z* 265, 297, 314, 473, the same as those of *M1* and *M2*, suggested that *M3* was the further sulfated conjugate of *M1* and *M2*. The sulfated and glucuronidated positions were therefore suggested to be at 1- or 9-hydroxyl group (Fig. 3B).

3.2.4. Metabolites M4 and M5

The metabolite *M4* was found in both rat urine and bile samples. *M4* was observed at retention time of 12.99 min. The protonated molecular ion of *M4* was at *m/z* 394 (*m/z* 314 + 80 Da), the MS^2 spectra of *M4* showed major product ions at *m/z* 265, 297, 377 and 314. The presence of the product ion at *m/z* 377 formed by the loss of an amino group from *m/z* 394 indicated that the sulfated position was located at 1- or 9-hydroxyl group (Fig. 3C). Apparently, the protonated molecular ion of *M4* was 80 Da (a sulfate unit) more than that of norisoboldine, suggesting that this metabolite was the sulfated conjugate of norisoboldine after oral administration to the rat.

M5 was observed at retention time of 12.63 min and found in rat bile samples only, with the protonated molecular ion at *m/z* 474 (*m/z* 314 + 80 + 80 Da). The protonated molecular ion of *M5* was 80 Da (a sulfate unit) more than that of *M4*. The MS^2 spectra of *M5* showed major product ions at *m/z* 265, 297, 314, 377 and 394. The MS^2 product ions at *m/z* 265, 297, 314 and 377 were consis-

Table 1
Assignment of carbon and proton signals of norisoboldine metabolites *M1* and *M2*.

No.	Carbon signals[a,b]			Proton signals[a,b,c]		
	Norisoboldine	*M1*	*M2*	Norisoboldine	*M1*	*M2*
1	151.9	152.6	152.1			
2	145.5	147.1	146.0			
3	117.2	117.7	118.0	6.64 (1H, s)	6.96 (1H, s)	6.80 (1H, s)
3a	122.2	125.1	122.7			
4	26.9	27.2	27.2	2.86 (1H, d, *J* = 13 Hz) 3.08 (1H, m)	2.98 (1H, m) 3.17 (1H, m)	2.99 (1H, br. d, *J* = 15.5) 3.13 (1H, m)
5	43.7	43.6	43.5	3.10 (1H, m) 3.70 (1H, m)	3.17 (2H, m)	3.58 (1H, m) 3.730 (1H, br. s)
6a	55.3	55.1	54.4	3.57 (1H, br. d, *J* = 4 Hz)	3.61 (1H, m)	3.30 (1H, br. d, *J* = 12.0 Hz)
7	34.7	34.5	34.5	2.58 (1H, dd, *J* = 14, 14 Hz)	2.63 (1H, m)	2.60 (1H, m)
				2.81 (1H, dd, *J* = 4, 4 Hz)	2.81 (1H, m)	2.76 (1H, br. d, *J* = 9.5 Hz)
7a	128.7	128.8	128.7			
8	117.0	117.2	114.7	6.64 (1H, s)	6.65 (1H, s)	6.82 (1H, s)
9	147.2	147.5	145.8			
10	148.9	148.9	148.8			
11	113.5	114.0	113.5	7.42 (1H, s)	7.52 (1H, s)	7.44 (1H, s)
11a	125.4	125.2	127.4			
11b	129.4	129.7	130.0			
11c	128.3	129.3	128.2			
2-OMe	62.2	63.0	62.3	3.61 (3H, s)	3.52 (3H, s)	3.46 (3H, s)
10-OMe	58.0	58.3	57.5	3.34 (3H, s)	3.64 (3H, s)	3.22 (3H, s)
1′[d]		102.6	99.4		5.23 (1H, d, *J* = 6.0 Hz)	5.36 (1H, d, *J* = 4.0 Hz)
2′		75.5	75.3		3.68–3.73 (1H, m)	3.73 (1H, br. s)
3′		79.4	79.0		3.91 (1H, br. d, *J* = 9.0 Hz)	4.03 (1H, d, *J* = 9.0 Hz)
4′		74.4	74.7		3.68–3.73 (1H, m)	3.64 (1H, dd, *J* = 8.5, 9.0 Hz)
5′		78.0	78.0		3.68–3.73 (1H, m)	3.73 (1H, br. s)
6′		176.9	178.5			

[a] All spectra were recorded on a Bruker AV 500 spectrometer, in D_2O.
[b] The carbon and proton signals were assigned on ^1H NMR, ^{13}C NMR, HMBC and HSQC.
[c] m, multiple split.
[d] Carbon number of glucuronic acid moiety.

tent with those ions observed in the MS^2 spectra of *M4* (Fig. 3C). This indicated that *M5* is a derivative of the norisoboldine with two sulfate groups added on to the 1- and 9-hydroxyl positions of the norisoboldine.

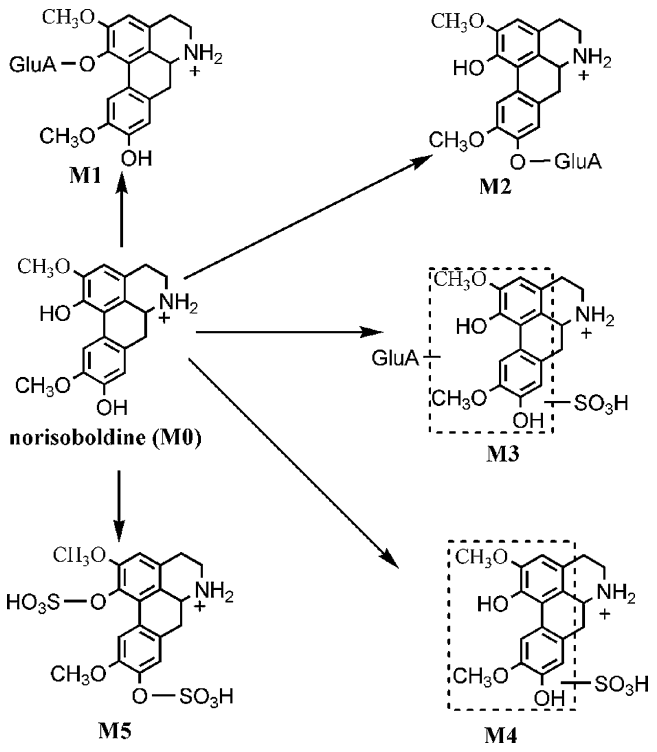

Fig. 4. The proposed metabolic pathways of norisoboldine in rat urine and bile samples after oral administration of 40 mg/kg norisoboldine.

3.3. Elucidation of the possible metabolic pathways

The biotransformation of norisoboldine was investigated for the first time using a combination of HPLC-UV/MS^n and NMR. The above results indicated that formation of these metabolites can be explained by two proposed pathways: (1) the *O*-glucuronide conjugation and (2) the *O*-sulfate conjugation. Based on the structures of these metabolites, the proposed major metabolic pathways of norisoboldine in rat are shown in Fig. 4.

4. Conclusions

In this study, the results of HPLC-UV/MS^n and NMR studies have provided very useful information for elucidating structures of unknown norisoboldine metabolites in both urine and bile samples. Three new metabolites (*M1*, *M2* and *M5*) were isolated and characterized as noriosboldine-1-*O*-β-D-glucuronide, norisoboldine-9-*O*-α-D-glucuronide and disulfuric acid-1, 9-norisoboldine ester. Metabolic pathways of norisoboldine were proposed accordingly. The results indicated that norisoboldine was mainly biotransformed *in vivo* by glucuronidation and sulfation in rats.

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