Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 236–243

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Characterization of metabolites of worenine in rat biological samples using liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 14 July 2009
Received in revised form 26 August 2009
Accepted 29 August 2009
Available online 4 September 2009

Keywords:
Worenine
Metabolite
LC–MS/MS
Rat
Biological sample

ABSTRACT

The *in vivo* and *in vitro* metabolites of worenine in rat were identified or characterized using a specific and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. *In vivo* samples including rat urine, feces, and plasma samples were collected after ingestion of 25 mg/kg worenine to healthy rats. The *in vivo* and *in vitro* samples were cleaned up by a solid-phase extraction procedure (C18 cartridges) and a liquid–liquid extraction procedure, respectively. Then these pretreated samples were injected into a reversed-phase C18 column with mobile phase of methanol–ammonium acetate (2 mM, adjusted to pH 3.5 with formic acid) (60:40, v/v) and detected by an on-line MS/MS system. As a result, at least twenty-seven metabolites and the parent medicine were found in rat urine after ingestion of worenine. Seven metabolites and the parent medicine were identified or characterized in rat feces. Three metabolites and the parent medicine were detected in rat plasma. One metabolite was found in the rat intestinal flora incubation mixture, and three metabolites were characterized in the homogenized liver incubation mixture. The main phase I metabolism of worenine in rat was dehydrogenization, hydrogenation, hydroxylation, and demethylene reactions, and that of phase II was sulfation and glucuronidation.

1. Introduction

Worenine (structure shown in Fig. 1, existed isomerism) is one of the bioactive components isolated from *Coptidis rhizoma* (Huanglian), a widely used Traditional Chinese Medicine, which has been used for centuries for the treatment of dysentery, hypertension, inflammation, and liver diseases [1,2]. Despite its important therapeutic value, its *in vivo* or *in vitro* metabolism is not clear yet. Up till now, the works only focused on the qualitative determination of worenine in plant [3,4].

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been proven to be a powerful analytical tool for the identification of medicine and its metabolites in biological matrices due to its high sensitivity and specificity [5–8]. It is especially suitable for the analysis of thermolabile, highly polar and non-volatile metabolites. MS/MS technique has made possible the acquisition of structurally informative data from protonated molecules of analytes of interest, even when they are not resolved chromatographically [9,10]. Structural elucidation of medicine metabolites using LC–MS/MS is based on the premise that metabolites retain the substructures of the parent medicine molecule. MS–MS product ion spectrum of each metabolite provides detailed substructural information of its structure. So, using the product ion spectrum of parent medicine as a substructural template, metabolites presented in crude mixtures may be rapidly identified and detected based on their changes in molecular masses (ΔM) and spectral patterns of product ions, even without standards for each metabolite [11,12].

For studying the metabolism of worenine comprehensively, this work presents the metabolism of worenine in rat urine, feces and plasma. The present study also involves incubation of worenine with intestinal flora and homogenized liver in order to clarify its *in vivo* and *in vitro* metabolic pathway. The parent medicine and its twenty-seven metabolites were detected in rat urine after administration of worenine orally. Seven metabolites and the parent medicine were identified or characterized in rat feces. Three metabolites and the parent medicine were detected in rat plasma. Only one metabolite was found in the rat intestinal flora incubation mixture, and three metabolites were characterized in the homogenized liver incubation mixture. All the results were reported for the first time. These results will be useful for future studies involving worenine, such as clinical therapy.

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doi:10.1016/j.jpba.2009.08.026

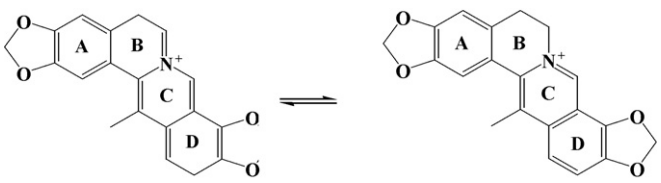

Fig. 1. The structure of worenine.

2. Experimental

2.1. Reagents and chemicals

Worenine was purchased from Beijing Hengye Zhongyuan Chemical Co., Ltd. (China). β-Glucuronidase (from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). Methanol was of HPLC grade (Fisher Chemical Co. Inc., CA, USA). Distilled water, prepared from demineralised water, was used throughout the study. Other reagents used were of analytical grade.

2.2. Instrumentation

LC–MS and LC–MS/MS experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer with a TSP4000 HPLC pump and a TSP AS3000 autosampler using positive electrospray as the ionization process (all components from Finnigan, Austin, TX, USA). A high-speed desk centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge samples. The urine samples were extracted on a C18 solid-phase extraction (SPE) cartridge (3 ml/200 mg, AccuBond, Agilent, Washington, DC, USA). The intestinal incubation experiments were carried out in anaerobic incubation bags (AnaeroPouchTM-Anaero 08G05A-23, Mitsubishi Gas Chemical Company Inc., Chiyoda-ku, Japan) using anaerobic-generating bags (Mitsubishi Gas Chemical Company Inc.).

2.3. Sample preparation

2.3.1. In vivo samples

Six male Wistar rats (200 ± 5 g, 2 months old, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine, feces and plasma. The rats were fasted for 24 h but with access to water, and then administered a single dose of worenine (25 mg/kg) by oral gavage. Urine samples were collected during the time period 0–48 h and centrifuged at 3000 × *g* for 10 min. Feces were collected individually during the time period of 0–24 h. The urine and feces samples were stored at −20 °C until analysis. Heparinized blood samples of 200 μl were collected at 1, 2, 4, 8, and 12 h from the ophthalmic veins of the rats by sterile capillary tube, then, shaken up and centrifuged at 2000 × *g* for 10 min. The supernatants were decanted, and immediately frozen at −20 °C until analysis.

2.3.1.1. Urine extraction. Free fraction. An aliquot of 1 ml of mixed 0–48 h urine samples was loaded onto a C18 SPE cartridge that was preconditioned with 2 ml of methanol and 1 ml of water. Then, the SPE cartridge was washed with 2 ml of water and the analytes were eluted with 1 ml of methanol. The effluent was filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

Acidic hydrolysis. After optimizing the acidity and the heated time, 0.8 ml of 6 M HCl and 50 mg of cysteine were added to 1 ml of mixed 0–48 h urine samples. The mixture was heated at 100 °C for 60 min. After cooling to room temperature, it was neutralized to pH 8 with 6 M NaOH and extracted with SPE cartridge immediately, just like the procedure mentioned above.

Enzymatic hydrolysis. After optimizing the acidity, temperature, enzymatic content and the time of hydrolysis, 1 ml of mixed 0–48 h urine samples was adjusted to pH 5.0 with a few drops of glacial acetic acid. Then, 0.5 ml of acetate buffer (0.5 mol/L, pH 5.0) and 0.2 ml of β-glucuronidase from *E. coli* (10,000 units/ml) were added to the solution prior to enzymatic hydrolyses. It took 5 h at 37 °C. After cooling, the solution was adjusted to pH 8 with 6 M NaOH and extracted with SPE cartridge immediately, just like the procedure mentioned above.

Free fraction was used for the comprehensive LC–MS/MS analyses of worenine and its metabolites. The target solutions after acidic and enzymatic hydrolyses were only used to assist in the investigation of phase II metabolites.

2.3.1.2. Feces and plasma extraction. The feces sample was homogenized with water. An aliquot of 500 μl feces homogenate was extracted twice with 1 ml of ethyl acetate after adding 50 μl of 0.001% Na_2CO_3 solution. The supernatant ethyl acetate layers were decanted, pooled and evaporated at 37 °C under nitrogen stream. The residue was redissolved in 500 μl of mobile phase and filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

The plasma samples were added 300 μl of methanol to precipitate plasma proteins, and then centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

2.3.2. In vitro samples

Preparation of anaerobic cultural solutions [13]. 37.5 ml A solution (0.78% K_2HPO_4), 37.5 ml B solution (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$, 0.25% $MgSO_4 \cdot H_2O$), 50 ml C solution (8% Na_2CO_3), 0.5 g L-cysteine, 2 ml 25% L-ascorbic acid, 1 g eurythrol, 1 g tryptone and 1 g nutrient agar were mixed together, and diluted to 1 l with distilled water. HCl (2 M) was used to adjust the solution to pH 7.5–8.0.

Metabolism in intestinal bacteria. The fresh intestinal contents were obtained from male Wistar rats (200 g, 2 months old). Samples were homogenized with a glass rod in anaerobic cultural solution at the rate of 0.5 g:1.5 ml immediately. Then, the homogenate was filtrated using gauze. Worenine was added into the above intestinal flora cultural solution to a final concentration of 50 μg/ml. The culture dishes were put in anaerobic incubation bags. The out bags of anaerobic-generating bags were opened, and put into anaerobic incubation bags immediately, then sealed. Incubations were carried out in a shaking water-bath at 37 °C anaerobically. The incubation was continued for 4 and 24 h, terminated and extracted (twice) with ethyl acetate. The organic extracts were merged and evaporated at 37 °C under nitrogen stream. The residues were reconstituted in 0.6 ml of mobile phase, centrifuged at 13,000 × *g* for 10 min. The supernatant was used for LC–MS/MS analyses.

Preparation and incubation of liver homogenate. Male Wistar rats (200 g, 2 months old) were fasted for 24 h and killed by decapitation between 10 a.m. and noon. A weighed amount of liver was rapidly placed on ice. It was rinsed twice with saline and immediately minced with scissors and homogenized in ice-cold Krebs–Henseleit buffer (pH 7.4) [14] after sterilization to yield liver homogenate (0.4 g/ml). All the above steps were carried out at 0–4 °C. The concentration of P450 was detected by spectrophotometry [15]. Worenine was added to liver homogenate at a concentration of 50 μg/ml. The mixture was incubated at 37 °C with shaking. The incubation time was varied from 0, 30, 60, 90, 120 to 240 min. The gas phase was oxygen. After the termination of incubation the mixture was extracted twice with equal volumes of ethyl acetate. The organic extracts were merged and evaporated at 37 °C under a slow

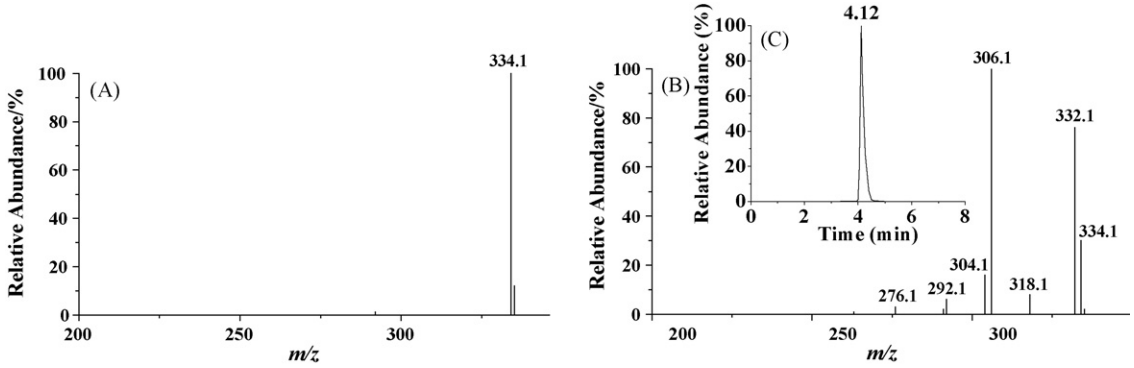

Fig. 2. Full scan MS spectrum (A), full scan MS^2 product ions spectrum (B) and LC–MS^2 chromatogram (C) of worenine.

stream of nitrogen. The residues were reconstituted in 0.6 ml of mobile phase, centrifuged at 13,000 × *g* for 10 min. The supernatant was used for LC–MS/MS analyses. The blank experiment was carried out under the same conditions by replacing the liver homogenate with Krebs–Henseleit buffer.

2.4. Chromatography and mass spectrometry

A reversed-phase column (Dionext-C18, 5 μm, 4.6 mm × 250 mm i.d., Sunnyvale, USA) was connected with a guard column (cartridge 2.1 mm × 12.5 mm, 5 μm, Dionex, Sunnyvale, CA, USA) filled with the same packing material to separate worenine and its metabolites. The temperature of the column was set at 25 °C. The mobile phase consisted of methanol and ammonium acetate (2 mM, adjusted to pH 3.5 with formic acid) (60:40, v/v). The flow rate was 0.2 ml/min during the whole run. Mass spectral analyses were carried out in positive ion detection mode. Nitrogen was used as the sheath gas (36 a.u.). The MS analyses were performed under automatic gain control conditions, using a typical source spray voltage of 3.0 kV, a capillary voltage of 12 V and a heated capillary temperature of 300 °C. The other parameters were also optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument. The MS/MS product ion spectra were produced by collision induced dissociation of the molecular ion $[M]^+$ of all analytes. Data acquisition was performed in full scan LC–MS and tandem MS modes.

3. Results and discussion

3.1. LC–MS and LC–MS/MS analyses of substrate

The chromatographic and mass spectrometry conditions were optimized with the use of worenine. Full scan mass spectral analysis of worenine showed the presence of $[M]^+$ (*m*/*z* 334) (Fig. 2A). The MS–MS product ion spectrum of worenine (*m*/*z* 334) was shown in Fig. 2B. Worenine was eluted at 4.12 min (Fig. 2C). Fragmentation of molecule of worenine in the ion trap lead to five product

Table 1
Comparison between different extraction procedures.

Analyte	$[M]^+$	t_R (min)	Peak area (×10^5)			A	B	C	D	E
			Free SPE fraction	Acidic fraction	Enzymatic fraction					
M0	334	4.12	332.0	340.2	338.1	+	+	+	+	+
M1	336	3.85	121.7	139.5	128.2	+	+	+	+	+
M2	338	3.66	100.5	134.6	114.2	+	+	–	–	–
M3	322	3.84	12.7	25.4	22.9	+	+	+	–	+
M4	324	3.49	19.4	35.0	32.7	+	+	–	–	–
M5	310	3.82	65.9	77.4	73.2	+	+	–	–	–
M6	350	3.66	32.1	39.0	36.1	+	+	+	–	+
M7	366	3.78	6.5	5.0	7.6	+	+	–	–	–
M8	382	3.80	7.0	7.5	7.1	+	–	–	–	–
M9	332	3.91	10.1	11.6	9.3	+	–	–	–	–
M10	320	3.65	12.3	20.7	16.1	+	–	–	–	–
M11	308	3.73	15.3	21.3	17.0	+	–	–	–	–
M12	388	3.38	1.8	–	1.1	+	–	–	–	–
M13	400	3.23	5.9	–	5.3	+	–	–	–	–
M14	416	3.46	8.2	–	8.2	+	–	–	–	–
M15	418	3.21	12.8	Trace	11.2	+	–	–	–	–
M16	484	3.32	0.4	–	–	+	–	–	–	–
M17	486	3.19	11.0	–	–	+	–	–	–	–
M18	496	3.27	3.3	–	–	+	–	–	–	–
M19	498	3.35	8.3	–	–	+	–	–	–	–
M20	500	3.60	6.2	–	–	+	–	–	–	–
M21	512	3.37	13.1	–	Trace	+	–	–	–	–
M22	514	3.29	17.2	Trace	Trace	+	–	–	–	–
M23	526	3.41	4.6	–	–	+	–	–	–	–
M24	660	3.11	1.8	–	–	+	–	–	–	–
M25	662	3.30	0.7	–	–	+	–	–	–	–
M26	674	3.52	3.0	–	–	+	–	–	–	–
M27	676	3.40	5.8	–	–	+	–	–	–	–

A: urine; B: feces; D: plasma; C: intestinal incubation; E: liver homogenate incubation; +: found; –: not found.

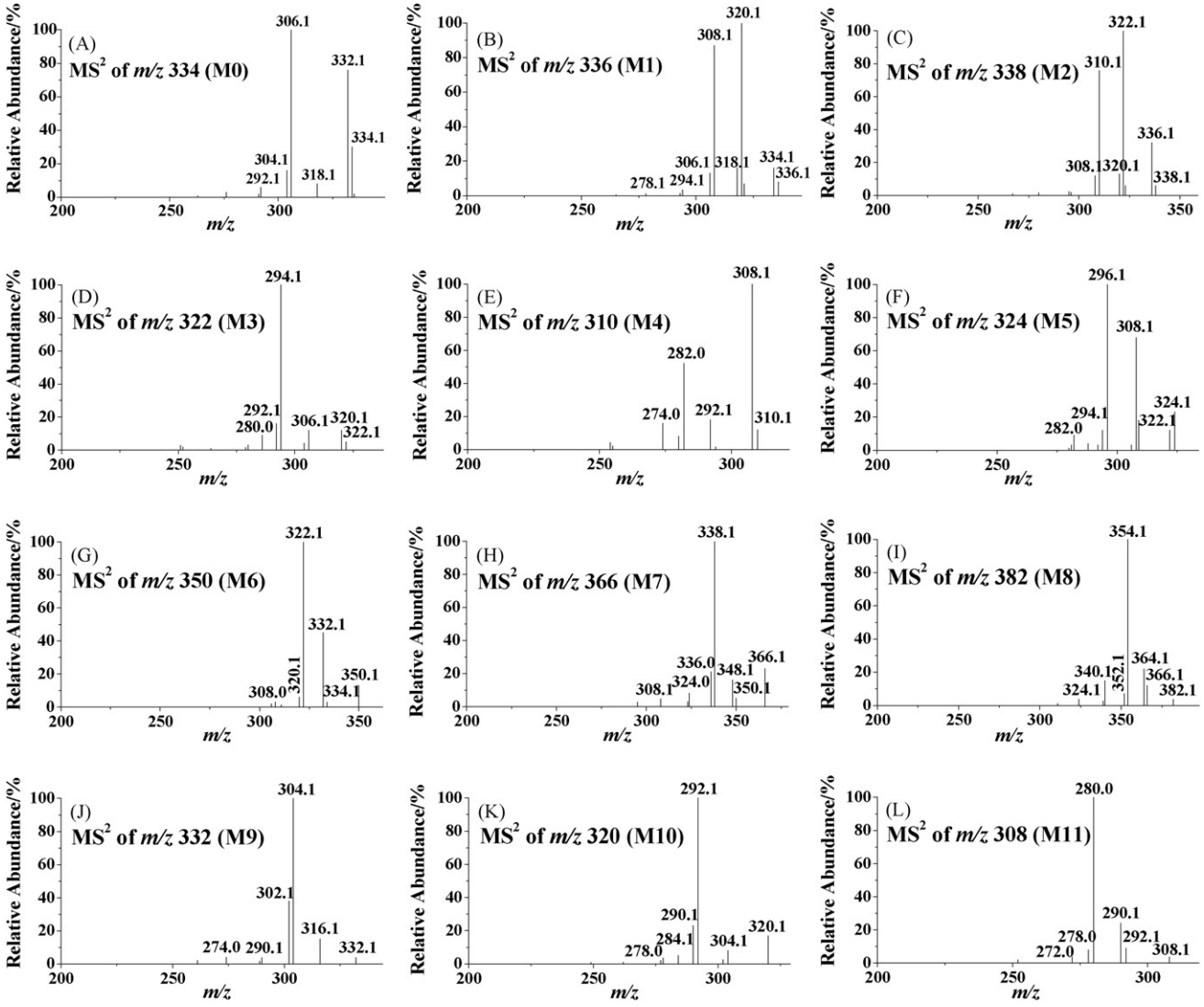

Fig. 3. MS^2 product ion spectra of worenine and its phase I metabolites in rat urine.

ions at *m*/*z*: 332, 318, 306, 304, 292 and 276. The most abundant product ion at *m*/*z* 306 was produced by the loss of CO (28 Da). The subordinate product ions at *m*/*z* 332 were formed by the loss of H_2 (2 Da). The product ion at *m*/*z* 304 was formed by the loss of CO + H_2 (30 Da). The product ion at *m*/*z* 292 was formed by the loss of CH_2 + CO (42 Da). The product ion at *m*/*z* 276 was formed by the loss of H_2 + 2CO (58 Da). It could be concluded that the ions at *m*/*z* 332, 318, 306, 304, 292 and 276 were the characteristic product ions of worenine. 2, 16, 28, 30, 42 and 58 Da were the characteristic neutral losses of worenine. These characteristic product ions and neutral losses were the sound bases to identify metabolites of worenine.

3.2. In vivo metabolism study

3.2.1. Characterization of metabolites in rat urine

The full scan mass spectrum of free fraction of rat urine after administration of worenine was compared with those of blank urine samples and worenine solution to find out the possible metabolites in rat urine. Then, these compounds were analyzed by LC–MS/MS. Their retention times, changes in observed mass and spectral patterns of product ions were compared with those of worenine standard to identify metabolites and elucidate their structures.

Based on the method mentioned above, the parent medicine and its main metabolites were found in rat urine (mixed 0–48 h urine samples) after administration of worenine. Their molecular ions ($[M]^+$) were at *m*/*z* 308, 310, 320, 322, 324, 332, 334, 336, 338, 350, 366, 382, 388, 400, 416, 418, 484, 486, 496, 498, 500, 512, 514, 526, 660, 662, 674 and 676, respectively. Their retention times (Table 1) were all shorter than that of worenine, which accorded with the metabolism rule of medicines.

LC–MS/MS spectra of the metabolites of worenine were obtained *via* fragmentation of molecular ions that used for more precise structural identification of metabolites. The MS^2 product ion spectra of these analytes were shown in Figs. 3 and 4. Among them, the retention time, the MS and MS^2 spectra of the molecular ion at *m*/*z* 334 (M0, Fig. 3A) were the same as those of worenine. Therefore, M0 could be confirmed as the unchanged parent medicine.

The molecular ion at *m*/*z* 336 (M1) and its product ions at *m*/*z* 334, 320, 308, 306, 294 and 278 (Fig. 3B) were all 2 Da more than *m*/*z* 334 and its product ions at *m*/*z* 332, 318, 306, 304, 292 and 276, respectively. Therefore, M1 should be the hydrogenation product

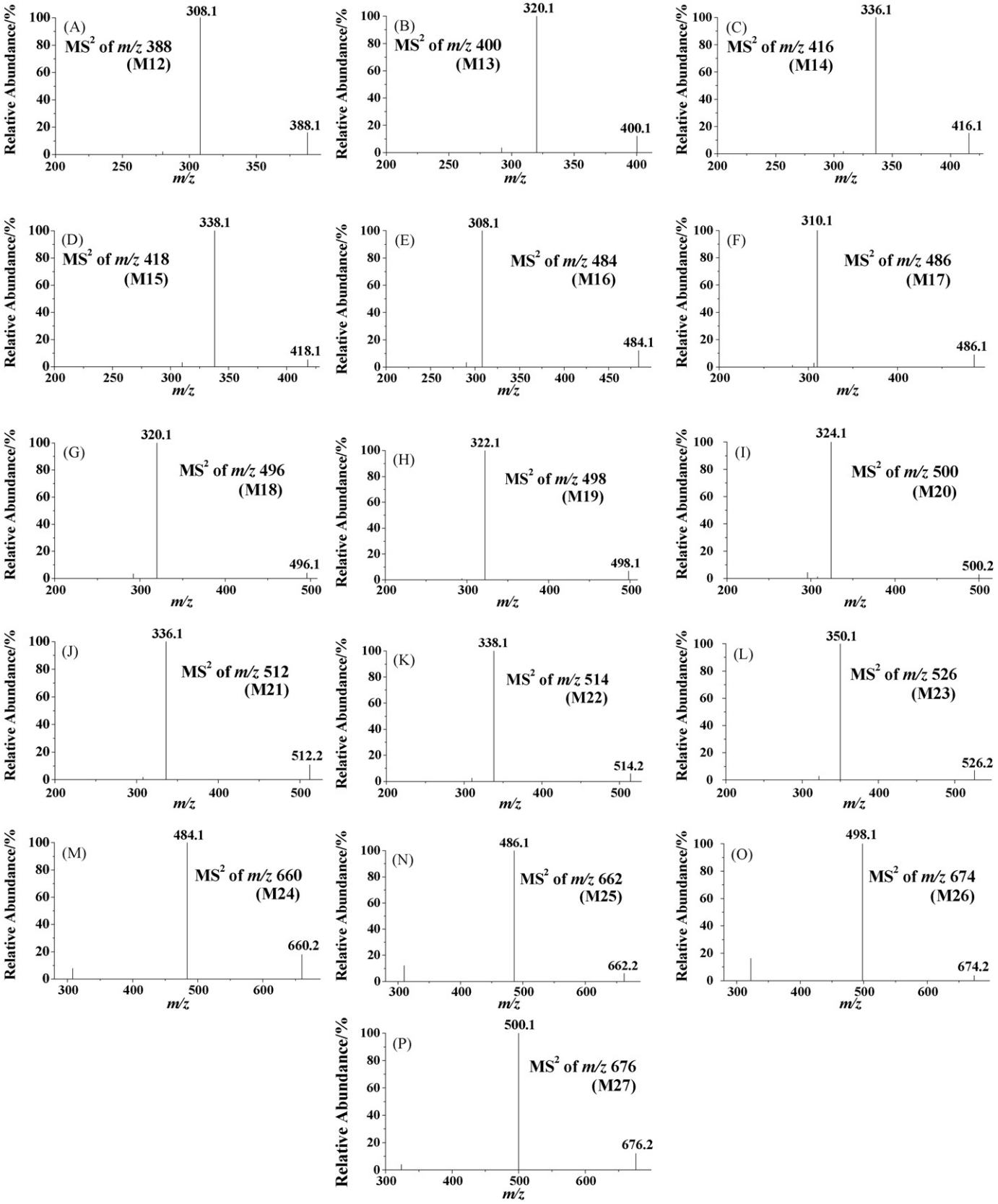

Fig. 4. MS^2 product ion spectra of phase II metabolites of worenine in rat urine.

of worenine, along with the breaking reaction of C–O bond of the 5-membered ring. The molecular ion at *m/z* 338 (M2) and its product ions at *m/z* 336, 322, 310, 308, 296 and 280 (Fig. 3C) were all 4 Da more than *m/z* 334 and its product ions at *m/z* 332, 318, 306, 304, 292 and 276, respectively. Therefore, M2 should be the hydrogenation product of worenine, along with the breaking reaction of C–O bond of the two 5-membered rings.

The molecular ion at *m/z* 322 (M3) and its product ions at *m/z* 320, 306, 294, 292 and 280 (Fig. 3D) were all 12 Da less than *m/z* 334 (M0) and its product ions at *m/z* 332, 318, 306, 304 and 292, respectively. Therefore, M3 should be the demethylene product of worenine. Also, the molecular ion at *m/z* 310 (M4) (Fig. 3E) was identified as the de-dimethylene product of worenine as the same reason.

The molecular ion at *m/z* 324 (M5) and its product ions at *m/z* 322, 308, 296 and 294 (Fig. 3F) were all 14 Da less than *m/z* 338 (M2) and its product ions *m/z* 336, 322, 310 and 308, respectively. Therefore, M5 should be the demethylated product of M2.

The molecular ion of M6 (*m/z* 350), M7 (*m/z* 366), M8 (*m/z* 382), along with the corresponding main fragment ions of them (Fig. 3G–I), were increased by 16, 32 and 48 Da, respectively, compared with those of the parent medicine. The results indicated that M6–M8 should be the mono-hydroxylate, di-hydroxylate and tri-hydroxylate of worenine, respectively. According to the structure of the parent medicine, the saturated carbon atom at B-ring is near to an *ortho* sp^2 hybridization C-atom at the A-ring and can be oxidized more easily than the carbon atoms at aromatic ring. Therefore, the first hydroxylation position may be the *meta*-position of the N-atom at the B-ring, the secondary hydroxylation position may be the saturated C-atom at the D-ring considering the isomer of the parent medicine (Fig. 1), and the third hydroxylation position may be the *ortho* position of the N-atom at the B-ring.

The molecular ion at *m/z* 332 (M9) and its product ions at *m/z* 316, 304, 302, 290 and 274 (Fig. 3J) were all 2 Da less than *m/z* 334 and its product ions at *m/z* 318, 306, 304, 292 and 276, respectively. Therefore, M9 was identified as the dehydrogenation product of worenine, and the dehydrogenated position was at the unique saturated C–C bond of the B-ring.

The molecular ions at *m/z* 320 (M10), 308 (M11) and their product ions (Fig. 3K and L) were all 12, 24 Da less than those of *m/z* 332 (M9), respectively. So, M10 and M11 could be characterized as demethylene product and de-dimethylene product of M9.

The MS^2 spectra of the molecular ion at *m/z* 388 (M12), 400 (M13), 416 (M14) and 418 (M15) showed the abundant product ions at *m/z* 308, 320, 336 and 338 (Fig. 4A–D), respectively, which were produced by neutral loss of 80 Da diagnostic of SO_3 from their molecular ions. And, the MS^3 spectra of *m/z* 388 → 308, 400 → 320, 416 → 336 and 418 → 338 were the same as the MS^2 spectra of M11, M10, M1 and M2, respectively. Based on these data, M12–M15 should be the sulfates of M11, M10, M1 and M2, respectively.

In the MS^2 spectra of *m/z* 484 (M16), 486 (M17), 496 (M18), 498 (M19), 500 (M20), 512 (M21), 514 (M22) and 526 (M23), the parent ions lost neutral fragment 176 Da to give its product ion at *m/z* 308, 310, 320, 322, 324, 336, 338 and 350 (Fig. 4E–L). The MS^3 spectra of *m/z* 484 → 308, *m/z* 486 → 310, *m/z* 496 → 320, *m/z* 498 → 322, *m/z* 500 → 324, *m/z* 512 → 336, *m/z* 514 → 338 and *m/z* 526 → 350 were the as the same as the MS^2 spectra of M11, M4, M10, M3, M5, M1, M2 and M6, respectively. So, M16, M17, M18, M19, M20, M21, M22 and M23 should be the glucuronides of M11, M4, M10, M3, M5, M1, M2 and M6, respectively.

The predominant product ion at *m/z* 484, 486, 498 and 500 was formed by the loss of neutral fragment 176 Da from the parent ion at *m/z* 660 (M24), 662 (M25), 674 (M26), 676 (M27) (Fig. 4M–P), respectively, and the MS^3 spectrum of *m/z* 660 → 484, 662 → 486, 674 → 498, 676 → 500 was the same as the MS^2 spectrum of M16, M17, M19 and M20, respectively. Therefore, M24–M27 should be the di-glucuronides of M11, M4, M3 and M5, respectively.

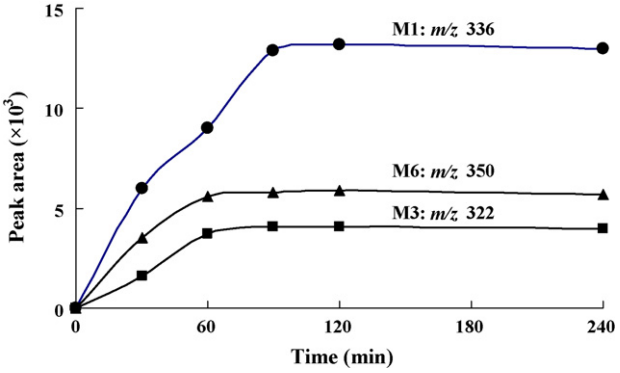

Fig. 5. Time–area curves of metabolites of worenine in homogenized liver of rats.

These metabolites can be investigated further by comparing various extraction techniques (Table 1). Compared with free fraction, the peak areas of M1–M6, M10 and M11 increased, and those of M12–M27 decreased obviously after acidic hydrolyses. The peak areas of M1–M6, M10 and M11 increased, and those of M16–M27 decreased obviously after enzymatic hydrolysis. These results revealed that M1–M6, M10 and M11 excreted from rat urine as the free, sulfate or glucuronide.

3.2.2. Characterization of metabolites in rat feces

The full scan mass spectrum of rat feces after injection of worenine was compared with that of blank feces sample and worenine solution to find out the possible metabolites. The parent medicine and its metabolites were found in rat feces after administration of worenine orally. Their molecular ions ($[M]^+$) were at *m/z* 310, 322, 324, 334, 336, 338, 350 and 366, respectively. They were characterized as M4, M3, M5, M0, M1, M2, M6 and M7, respectively.

3.2.3. Characterization of metabolites in rat plasma

Comparing with blank plasma sample and worenine standard, the parent medicine and its three metabolites were detected in rat plasma after injection of worenine. Their molecular ions ($[M]^+$) were at *m/z* 322, 334, 336 and 350, respectively. They were characterized as M3, M0, M1 and M6, respectively.

The LC–MS/MS analysis of plasma samples showed that the parent medicine and its three metabolites (M1, M3, M6) all existed in plasma between 1 and 12 h after administration.

The phase II metabolites of worenine were not found in rat plasma or feces.

3.3. In vitro metabolism study

The substrate was incubated with intestinal fractions and liver homogenate of rats, respectively. Comparing with blank sample and worenine standard, the substrate (M0) and M1 were detected in rat intestinal part and, M0, M1, M3 and M6 were found in liver homogenate part. The results were almost the same when intestinal incubation solutions at 4 and 24 h were investigated. Fig. 5 presented the time–area curves of metabolites of worenine in homogenized liver of rats.

The above results showed that the metabolism of worenine in rat intestine was very weak. The metabolism of worenine should mainly occur in rat liver. The study on the *in vitro* metabolism could expound the *in vivo* position where the metabolism of worenine occurred.

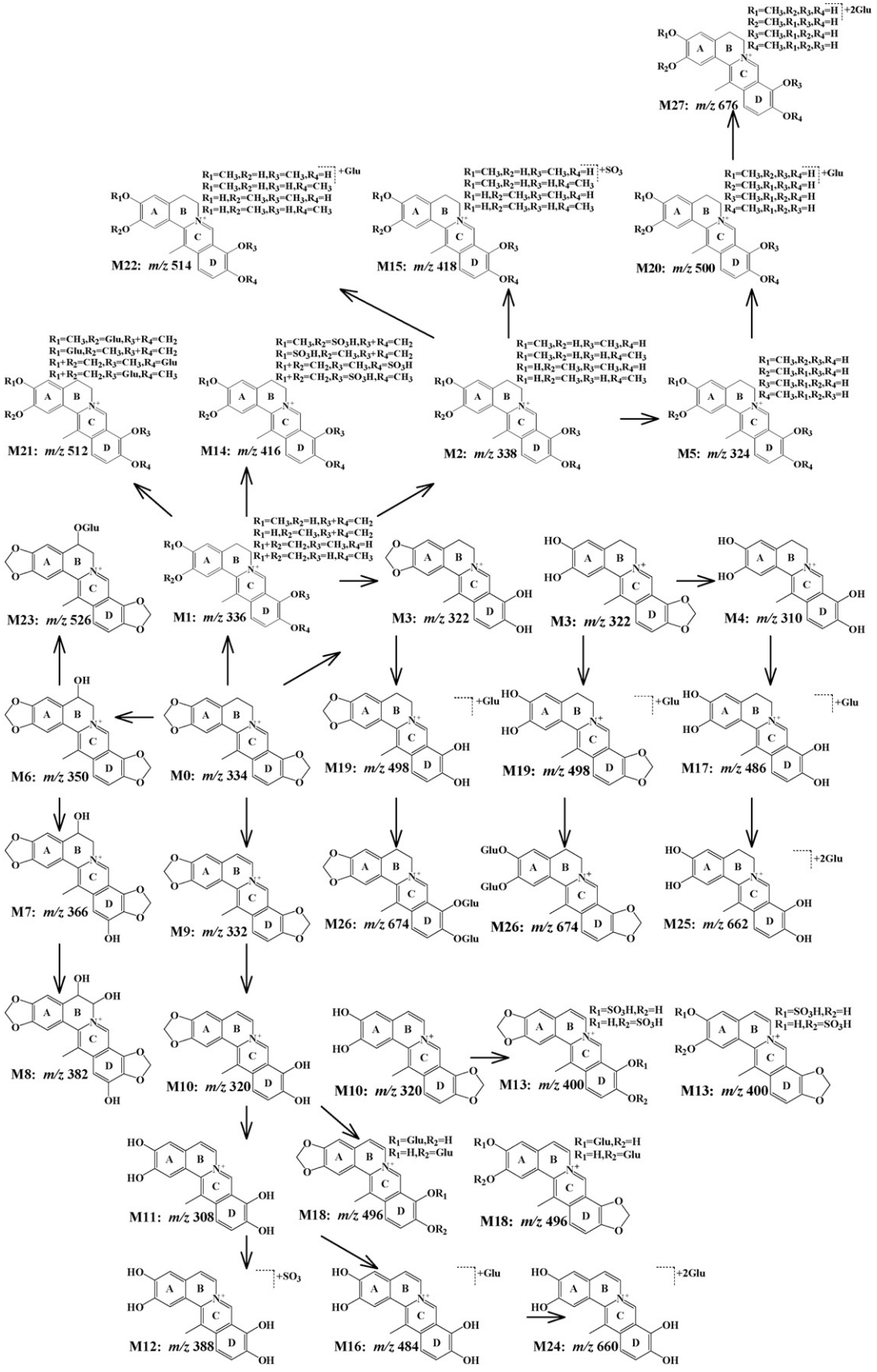

Fig. 6. Proposed major metabolic pathway of worenine in rat (Glu: glucuronic acid).

3.4. Validation study

The sensitivity of the method was determined for worenine standard and the limit of detection (LOD, S/N = 3) was found to be below 2 ng ml^{-1} by LC–MS^2. For the free fraction of rat urine, mean recoveries (n = 5) were 88.7% at concentrations of 5 ng ml^{-1}. The specificity of the assay was evaluated by analyzing blank urine, feces or plasma samples from rats; no impurities or endogenous interferences were found.

4. Conclusions

For the first time, *in vivo* and *in vitro* metabolism of worenine in rats was studied extensively. Metabolites were resolved, identified or characterized using LC–MS/MS ion trap with electrospray ionization in the positive ion mode. Twenty-seven metabolites were found in rat urine and seven, three, one, and three metabolites were characterized in rat feces, plasma, intestinal bacteria and liver homogenate, respectively. The proposed metabolic pathway of worenine in rats was presented in Fig. 6. M4, M9 and M11 were identified compounds. And, others were characterized compounds. M1–M11 were the phase I metabolites of worenine in rat. The phase II metabolites of worenine in rat included four sulfates (M12–M15), eight glucuronides (M16–M23) and four diglucuronides (M24–M27). The experimental results showed that the main phase I metabolism of worenine in rat was dehydrogenization, hydrogenation, hydroxylation, and demethylene reactions, and the phase II metabolism of worenine in rat was sulfation and glucuronidation. Though the structures of metabolites cannot be determined conclusively by LC–MS/MS alone, the present method is still very valuable and dependable for the further study of the metabolism of worenine.

Acknowledgements

This work was supported by the Key (Keygrant) Project of Chinese Ministry of Education (No. 208090), the Ministry-of-Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules Natural Science Foundation of Hubei University (No. 020-044109) and the Specialist Fund of Hubei University (020091130-ky2006004). The authors would like to thank their colleagues for their valuable technical assistance.

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