JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 647–652

Received November 5, 2008
Accepted January 12, 2009
DOI 10.1211/jpp/61.05.0014
ISSN 0022-3573

Research Paper

Identification of in-vivo and in-vitro metabolites of palmatine by liquid chromatography–tandem mass spectrometry

Qian C. Yang, Wen H. Wu, Feng M. Han and Yong Chen

Hubei Province Key Laboratory of Biotechnology of Chinese Traditional Medicine, Hubei University, Wuhan, China

Abstract

Objectives Despite its important therapeutic value, the metabolism of palmatine is not yet clear. Our objective was to investigate its in-vivo and in-vitro metabolism.

Methods Liquid chromatography–tandem electrospray ionization mass spectrometry (LC-ESI/MS^n) was employed in this work. In-vivo samples, including faeces, urine and plasma of rats, were collected after oral administration of palmatine (20 mg/kg) to rats. In-vitro samples were prepared by incubating palmatine with intestinal flora and liver microsome of rats, respectively. All the samples were purified via a C_{18} solid-phase extraction procedure, then chromatographically separated by a reverse-phase C_{18} column with methanol–formic acid aqueous solution (pH 3.5, 70 : 30 v/v) as mobile phase, and detected by an on-line MS^n detector. The structure of each metabolite was elucidated by comparing its molecular weight, retention time and full-scan MS^n spectra with those of the parent drug.

Key findings The results revealed that 12 metabolites were present in rat faeces, 13 metabolites in rat urine, 7 metabolites in rat plasma, 10 metabolites in rat intestinal flora and 9 metabolites in rat liver microsomes. Except for six of the metabolites in rat urine, the other in-vivo and in-vitro metabolites were reported for the first time.

Conclusions Seven new metabolites of palmatine (tri-hydroxyl palmatine, di-demethoxyl palmatine, tri-demethyl palmatine, mono-demethoxyl dehydrogen palmatine, di-demethoxyl dehydrogen palmatine, mono-demethyl dehydrogen palmatine, tri-demethyl dehydrogen palmatine) were reported in this work.

Keywords LC-MS^n; palmatine; palmatine metabolites; rat

Introduction

Palmatine (Figure 1)[1] is an isoquinoline alkaloid extracted from the Chinese herb *Fibaurea recisa* Pierre. There has been much research into its biological activity, such as its antibacterial, antifungal and antiviral activity,[2,3] and its sedative,[4] vasodilatory[5] and hepatoprotective effect.[6] Despite its important therapeutic value, its in-vivo and in-vitro metabolism is not yet clear. Many quantitative methods have been developed to determine the content of palmatine, including HPLC,[7] CE-MS,[8] fluorescence quenching[9] and LC-MS.[10,11] Only one publication from our group has dealt with the identification of palmatine and its eight metabolites (designated as M1, M2, M4, M6, M7, M9 and glucuronide conjugates of M6 and M7) in rat urine after dosing rats with palmatine.[1] In this study, the metabolites of palmatine were identified in rat faeces, urine and plasma, as well as in intestinal flora and liver microsomes, after dosing Wistar rats with palmatine. The purpose of this work was to clarify its in-vivo and in-vitro metabolic pathway. This was very useful for further investigation of the metabolic kinetics and metabolism-based drug–drug interactions of palmatine.

Materials and Methods

Chemicals and reagents

Palmatine hydrochloride was purchased from Sigma (St Louis, MO, USA). HPLC-grade methanol was from Fisher Chemical Co., Inc. (Pittsburgh, PA, USA). Water was de-ionized and double distilled. All other reagents used were of analytical grade.

Correspondence: Professor Yong Chen, Department of Life Science, Hubei University, Wuhan 430062, China.
E-mail: cy101610@yahoo.cn

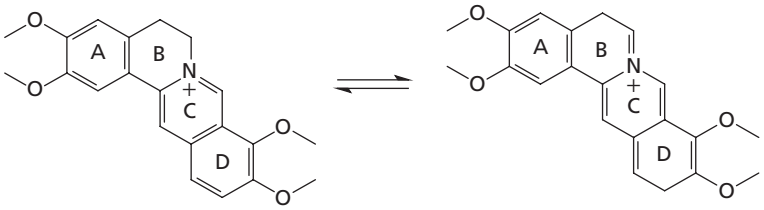

Figure 1 The structure of palmatine[1]

Analysis conditions

Liquid chromatography–mass spectrometry (LC-MS^n) experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (Thermo-Finnigan Corp, San Jose, CA, USA) equipped with Agilent 1100 Series G1311A Quat pump and G1313A autosampler. The software Xcalibur version 1.2 (Thermo-Finnigan Corp) was applied for the system operation and data collection. A high-speed desk centrifuge (TGL-16C; Shanghai Anting Scientific Instrument Factory, Shanghai, China) and an ultracentrifuge (Optima LE-80K, Beckman Coulter, Fullerton, CA, USA) were used to centrifuge samples. A C_{18} solid-phase extraction (SPE) cartridge (3 ml/200 mg, AccuBond; Agilent, Santa Clara, CA, USA) was used for sample purification.

A reverse-phase column (Zorbax Extend-C_{18}, 2.1 mm × 50 mm i.d, 3.5 μm; Agilent) was connected to a guard column (4.6 mm × 12.5 mm cartridge, 5 μm; Agilent) to separate palmatine and its metabolites. The column temperature was set at 25°C. The mobile phase was methanol–formic acid aqueous solution (70 : 30 v/v, pH 3.5). The flow rate was 0.2 ml/min during the whole chromatographic run time of 5 min. The injection volume of the sample was 20 μl.

Mass spectrometry was performed in electrospray ionization (ESI) positive ion mode. The operating conditions for the mass measurements were optimized by direct infusion into the MS detector of 20 μl palmatine solution (25 μg/ml dissolved in mobile phase) and chosen as follows: ion spray voltage, 4.5 kV; capillary voltage, 26 V; capillary temperature, 250°C; sheath gas flow rate, 80 arbitrary units; and auxiliary gas flow rate, 20 arbitrary units. Other parameters, including the voltages of octa-pole offset and tube lens offset were also optimized for maximum abundance of the molecular ion of palmatine, M^+, by the automatic tune procedure of the instrument. By isolating the molecular ions of metabolites in the ion trap, the product ion spectra were obtained by collision-induced dissociation (CID). The energy of CID was 35%. The selected reaction monitoring mode was used to determine the retention times of the parent drug and its metabolites. Data acquisition was performed in full scan LC-MS and tandem MS^n modes.

In-vivo sample preparation

Animals were managed according to the rules and regulations of the Institutional Animal Care and Use Committee at Hubei University, which also approved the experimental protocol.

Five Wistar rats (male and female, 180 ± 5 g; Hubei Experimental Animal Research Center, Wuhan, China) were housed in metabolic cages at optimal conditions of temperature (25 ± 2°C), humidity 60 ± 5% and a 12-h light–dark cycle with free access to food and water for one week; rats were then fasted for 48 h but allowed access to water before drug administration. All procedures were carried out in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals. Faeces and urine were collected during the time period of 0–48 h and 0–24 h, respectively, after administration of a single dose of palmatine (20 mg/kg) to rats by oral gavage, and stored at −20°C until analysis. Heparinized blood samples of 1 ml were collected at 1, 2, 4 and 8 h from the ophthalmic veins of the rats into sterile capillary tubes, then shaken up and centrifuged at 10 000*g* for 10 min. The supernatants were decanted and immediately frozen at −20°C until analysis.

The faeces sample collected at 0–48 h was homogenized with water (1 : 4 w/v) and then centrifuged at 5000*g* for 10 min. The supernatant was loaded onto a solid phase extraction (SPE) cartridge, which was preconditioned with 2 ml of methanol and 1 ml of water. Then, the SPE cartridge was washed with 2 ml of water and 1 ml of methanol in turn. The methanol eluent was centrifuged at 10 000*g* for 10 min. The supernatant was used for LC–MS^n analyses.

A volume of 1 ml of the urine sample collected at 0–24 h was purified by the SPE procedure as described above.

The blood sample collected at 1–8 h was treated by adding an equal volume of methanol to precipitate plasma proteins and purified by the SPE procedure as described above.

In-vitro sample preparation

Rat intestinal flora

The fresh faeces of rats were immediately homogenized with a glass rod in anaerobic culture solution in a ratio 1 : 4 (w/v), and then filtered through gauze. Intestinal flora were incubated in anaerobic culture solution (37.5 ml of solution A (0.78% K_2HPO_4), 37.5 ml of solution B (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$ and 0.25% $MgSO_4 \cdot H_2O$), 50 ml of solution C (8% Na_2CO_3), 2 ml of 25% L-ascorbic acid, 0.5 g L-cysteine, 1 g beef extract, 1 g tryptone and 1 g nutrient agar mixed together and diluted to 1 L with distilled water).[12] HCl (2 mol/l) was used to adjust the anaerobic culture solution to pH 7.5–8.0. Intestinal incubation experiments were performed in anaerobic incubation bags (AnaeroPouch Anaero 08G05A-23; Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) and anaerobic generating bags (Mitsubishi Gas Chemical Company Inc.).

Palmatine hydrochloride was added to the above intestinal flora culture solution to a final concentration of 0.1 mg/ml. The culture dishes were placed in the anaerobic incubation bags. Anaerobic incubation was carried out in a shaking water-bath at 37°C for 24 h, and then the incubation solution was centrifuged at 5000*g* for 10 min. The supernatant was purified by the SPE procedure as described above.

Rat liver microsomes

To minimize degradation of cytochrome P450, all apparatus and solutions were cooled and stored at 4°C before the experiment, and the whole preparation process of the rat liver microsomes was maintained at 4°C. Rats were weighed and killed by cervical dislocation. Liver tissue was washed with ice-cold normal saline, finely minced and homogenized with a four-fold volume of 0.1 mol/l phosphate-buffered saline (PBS) (pH 7.4). The homogenate was centrifuged at 10 000*g* for 20 min and the resulting supernatant was ultracentrifuged at 100 000*g* for 60 min to obtain the microsomal pellet. The washed pellet was re-suspended in 0.1 mol/l PBS (pH 7.4, containing 30% glycerin) and immediately stored at −80°C. The protein concentration was determined using Coomassie Brilliant Blue.

A 0.5-ml volume of the liver microsome incubation solution (containing 0.1 mol/l PBS (pH 7.4), 1 mg/ml microsomal protein, 5 mmol/l $MgCl_2$ and 0.25 mg/ml palmatine) was pre-incubated at 37°C for 3 min. The reaction was initiated by adding NADPH to a final concentration of 1.0 mmol/l, maintained at 37°C in a water bath shaker for 60 min and terminated by adding an equal volume of ice-cold methanol to precipitate proteins. The precipitate was removed by centrifugation at 10 000*g* for 10 min. The supernatant was evaporated at 37°C under a nitrogen stream. The residue was re-dissolved in 1 ml of methanol, then centrifuged at 10 000*g* for 10 min. The supernatant was purified by the SPE method as described above.

Structural analysis of palmatine metabolites

Firstly, possible structures for the metabolites of palmatine were postulated in accordance with the parent structure and the known common metabolic pathways. Then, the full scan mass spectra of the purified samples from the drug-treated rats were compared with those of the corresponding control rats to find the possible metabolites. No endogenous impurity interference and obvious ion suppression should be found in the purified samples. Subsequently, the MS^n spectra of the possible metabolites were obtained by CID through isolating the molecular ion or the fragment ion of interest in the ion trap, respectively. Finally, structural elucidation of the metabolite was performed by comparing its mass spectral patterns with those of the parent drug.

Results

LC–MS^n analysis of palmatine

The mass spectral behaviour of palmatine provides important information for identification and structural elucidation of its metabolites and has been discussed in our earlier work.[1] Under the experimental conditions used here, palmatine was eluted at 2.69 min (Figure 2c). The full scan mass spectrum of palmatine gave the molecular ion, M^+, at *m/z* 352 in ESI positive ion mode (Figure 2a). The fragmentation of palmatine in the ion trap led to three MS^2 product ions at *m/z* 337, 322 and 308 (Figure 2b). The fragment ion at *m/z* 294 was present in the MS^3 spectra of the ions at *m/z* 337, 322 and 308. The characteristic fragment ions of palmatine at *m/z* 337, 322, 308 and 294 were formed as shown in Figure 3.

Identification of metabolites *in vivo*

Metabolites in rat urine

After drug administration, the parent drug and its thirteen metabolites (Figure 4) were detected in rat urine with the molecule ions (M^+) at *m/z* 290, 292, 308, 310, 320, 322, 324, 336, 338, 350, 352, 368, 384 and 400, respectively. Among them, the retention time and the MS, MS^2 and MS^3 spectra of the molecular ion at *m/z* 352 (M0, T = 2.69 min) were the same as those of palmatine. Therefore, M0 was confirmed as the unchanged parent drug.

The molecular ion of M1 (*m/z* 368, T = 2.46 min) and its product ions at *m/z* 324, 338 and 353 were all 16 Da more than those of M0, respectively. Therefore, M1 was confirmed as the mono-hydroxylate of M0. The molecular ion of M2 (*m/z* 384, T = 2.31 min) and its product ions at *m/z* 369 and 340 were all increased by 32 Da compared with the molecular ion of M0 and its product ions at *m/z* 337 and 308, respectively. So, M2 should be the di-hydroxylate of M0. The molecular ion of M3 (*m/z* 400, T = 1.99 min) and its product ions at *m/z* 370 and 385 were all increased by

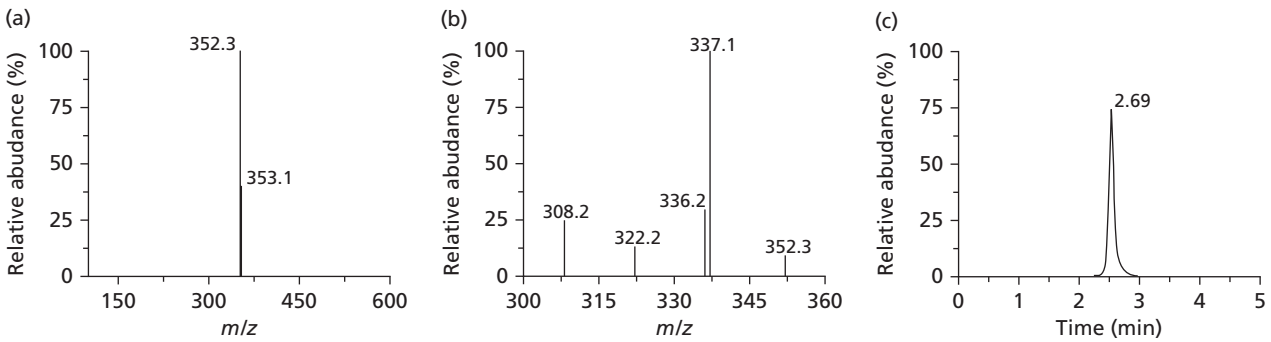

Figure 2 MS (a), MS^2 (b) and chromatogram (c) of palmatine

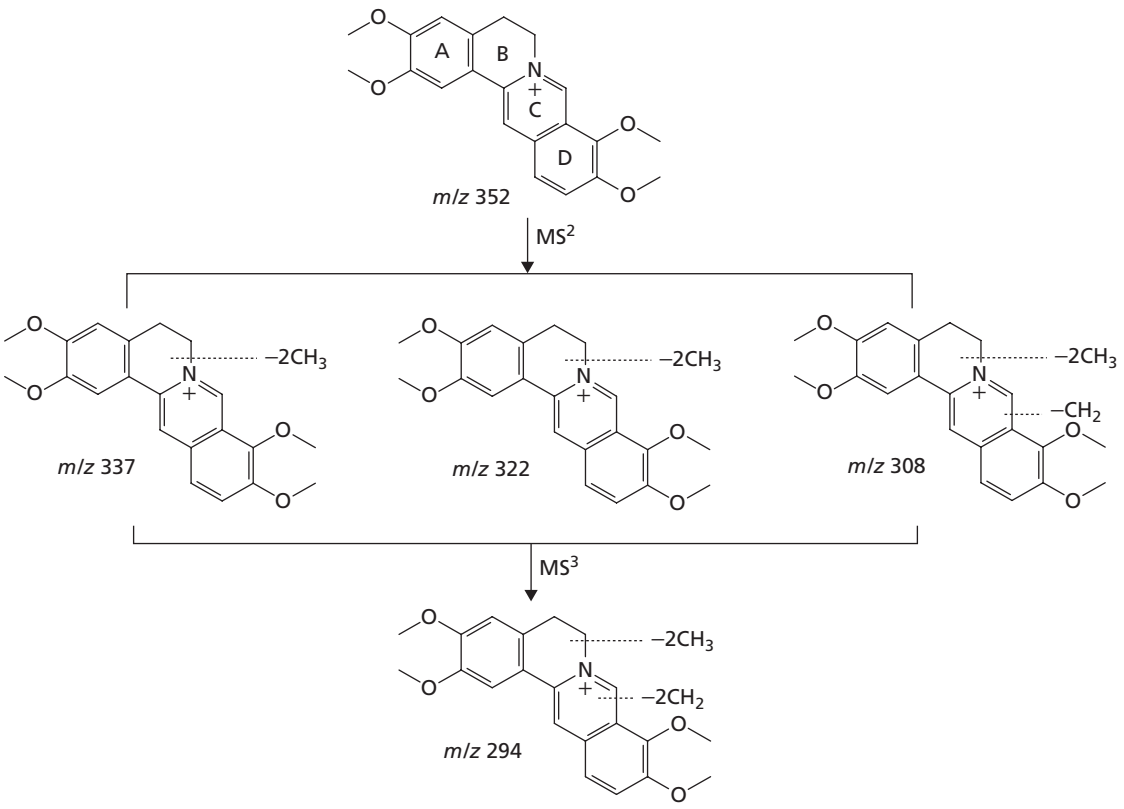

Figure 3 Proposed ESI-MS fragmentation pathways of palmatine in positive ion mode

48 Da compared with the molecular ion of M0 and its product ions at *m/z* 322 and 337, respectively. These results indicated that M3 should be the tri-hydroxylate of M0. Based on the known common oxidation metabolic reaction of drugs, the hydroxylation of the saturated aliphatic ring is easier than that of the aromatic ring, especially for the saturated carbon atom near to an *ortho* SP^2 hybridized carbon atom or a hetero-atom, such as an oxygen atom and nitrogen atom. According to the structure of the parent drug, the B ring and D ring of the conformer of the parent drug possess one saturated carbon atom, respectively (Figure 1). So, the first hydroxylation position should be the *meta* saturated carbon of the nitrogen atom at the B ring, the secondary hydroxylation position should be the saturated carbon atom at the D ring of the conformer of the parent drug and the third hydroxylation position should be the *ortho* saturated carbon of the nitrogen atom at the B ring.

The molecular ion of M4 (*m/z* 322, T = 2.37 min) led to four product ions at *m/z* 292, 293, 307 and 320. The product ion at *m/z* 320 was formed by the loss of 2H (2 Da) from the unique saturated C–C bond at the B ring of the molecular ion of M4, and the product ion at *m/z* 293 presumably resulted via the simultaneous loss of CH_2 (14 Da) and CH_3 (15 Da) from the molecular ion of M4. The molecular ion of M5 (*m/z* 292, T = 0.95 min) led to three main product ions at *m/z* 262, 277 and 290. The product ion at *m/z* 290 was formed by the loss of 2H (2 Da) from the unique saturated carbon bond at the B ring of the molecular ion of M5. The ions of M4 (*m/z* 322→*m/z* 292 and 307) and M5 (*m/z* 292→*m/z* 262 and 277) were all 30 Da and 60 Da less than the corresponding ions of M0 (*m/z* 352→*m/z* 322 and 337), respectively. Therefore, M4 and M5 should be the mono- and di-demethoxyl products of M0, respectively.

The molecular ion of M6 (*m/z* 338, T = 1.78 min) and its product ions at *m/z* 294, 308 and 323 were all 14 Da less than those of M0, respectively. The molecular ion of M7 (*m/z* 324, T = 1.13 min) and its product ions at *m/z* 294 and 309 were all 28 Da less than the molecular ion of M0 and its product ions at *m/z* 322 and 337, respectively. The molecular ion of M8 (*m/z* 310, T = 2.56 min) and its product ion at *m/z* 295 were all 42 Da less than the molecular ion of M0 and its product ion at *m/z* 337, respectively. The product ion at *m/z* 294 was formed by loss of an oxygen atom (16 Da) from the molecular ion of M8. So, M6, M7 and M8 can be confirmed as the mono-, di- and tri-demethylation products of M0, respectively.

The molecular ion at *m/z* 350 (M9, T = 1.94 min) and its product ions at *m/z* 306, 320 and 335 were all 2 Da less than those of M0, respectively. These results indicated that M9 should be the dehydrogenation product of M0, and the dehydrogenation position was at the unique saturated carbon bond at the B ring of M0.

The molecular ion of M10 (*m/z* 320, T = 0.87 min) and its product ions at *m/z* 276, 290 and 305 were decreased by 30 Da, respectively, compared with those of M9. The molecular ion of M11 (*m/z* 290, T = 0.73 min) and its product ions at *m/z* 260 and 275 were all 60 Da less than the molecular ion of M9 and its product ions at *m/z* 320 and 335.

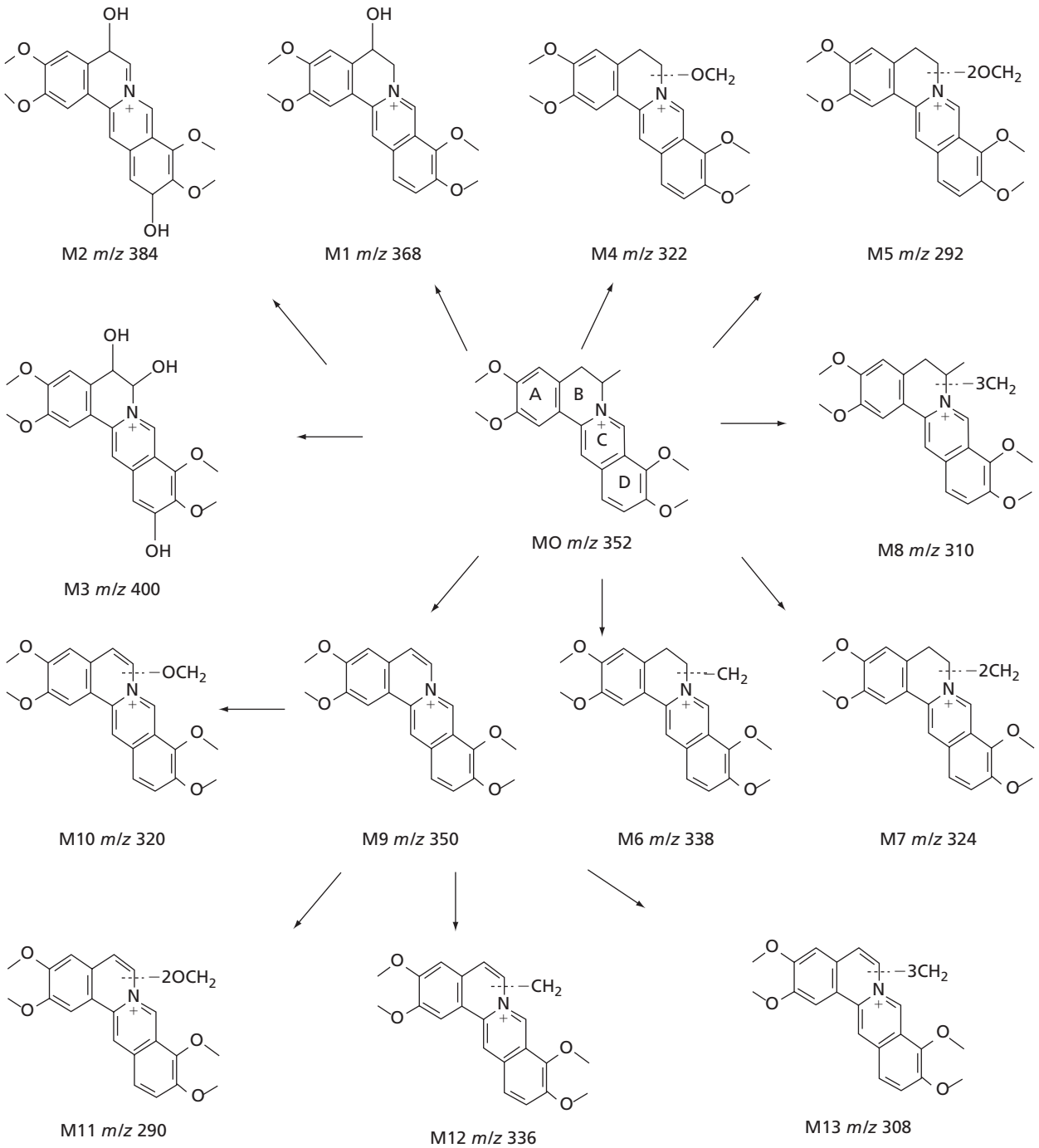

Figure 4 Proposed metabolic pathways of palmatine in rats

The predominant product ion at *m/z* 262 was formed by the loss of di-methylene (28 Da) from the molecular ion of M11 (*m/z* 290). Therefore, M10 and M11 should be the mono- and di-demethoxyl products of M9, respectively.

The molecular ion of M12 (*m/z* 336, T = 2.52 min) and its product ions at *m/z* 292, 306 and 321 were all 14 Da less than that of M9, respectively. The molecular ion of M13 (*m/z* 308, T = 2.21 min) and its product ion at *m/z* 293 were 42 Da less than the molecular ion of M9 and its product ion at *m/z* 335, respectively. These results indicated that M12 and M13 should be the mono- and tri-demethylation products of M9, respectively.

Metabolites in rat faeces

After drug administration, the parent drug and its twelve metabolites were detected in rat urine with the molecular ions (M^+) at *m/z* 290, 292, 308, 310, 320, 322, 324, 336, 338, 350, 352, 368 and 384. Their structures were elucidated the same

way as stated above. Except for metabolite M3, the others in rat urine were also detected in rat faeces.

Metabolites in rat plasma

There were seven metabolites of palmatine detected in rat plasma after drug administration. Their molecular ions (M^+) were at *m/z* 368, 322, 292, 338, 324, 320 and 336, and were identified as M1, M4, M5, M6, M7, M10 and M12, respectively, based on their MS^2 product ions.

In-vitro metabolism study

There were 10 metabolites of palmatine (M1, M4, M5, M6, M7, M8, M10, M11, M12 and M13) detected in the rat intestinal flora incubation solution, and nine metabolites of palmatine (M1, M4, M5, M6, M7, M8, M11, M12 and M13) detected in the liver microsome incubation solution.

Discussion

Tandem mass spectrometry (MS/MS) can provide much greater specificity than single-stage MS, allowing truly selective detection. Such specific detection provides greater confidence in assays and reduces the requirement for efficient separation. Although the technique of electrospray ionization (ESI) provides less structural information than electron ionization (EI), ESI ion-trap multistage mass spectrometry can also provide abundant structural information about analytes. The presented method in this paper is sensitive and specific for the identification and structural elucidation of palmatine and its metabolites. Seven new metabolites of palmatine (tri-hydroxyl palmatine, di-demethoxyl palmatine, tri-demethyl palmatine, mono-demethoxyl dehydrogen palmatine, di-demethoxyl dehydrogen palmatine, mono-demethyl dehydrogen palmatine, tri-demethyl dehydrogen palmatine) were reported in this paper. Except for the six metabolites in rat urine (mono-hydroxyl palmatine, di-hydroxyl palmatine, mono-demethoxyl palmatine, mono-demethyl palmatine, di-demethyl palmatine, dehydrogen palmatine), the other in-vivo and in-vitro metabolites of palmatine are reported here for the first time.

Conclusions

Based on the above discussion, the proposed metabolic pathway of palmatine in rats is shown in Figure 4. All of the metabolites are phase I metabolites. Although the structures of some metabolites can not be confirmed entirely by the present method alone, this work is still very valuable for providing understanding of the metabolism of palmatine *in vivo* and *in vitro*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This paper was supported by the National Natural Science Foundation of China (No. 30630075) and the Ministry of Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules (Hubei University) (No. 2007-KL-005).

Acknowledgements

The authors wish to thank their colleagues for their valuable technical assistance.

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