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Research Article

In vitro metabolism of corydaline in human liver microsomes and hepatocytes using liquid chromatography-ion trap mass spectrometry

Corydaline is a pharmacologically active isoquinoline alkaloid isolated from *Corydalis* tubers. It exhibits the antiacetylcholinesterase, antiallergic, antinociceptive, and gastric emptying activities. The purposes of this study were to establish *in vitro* metabolic pathways of corydaline in human liver microsomes and hepatocytes by identification of their metabolites using liquid chromatography-ion trap mass spectrometry. Human liver microsomal incubation of corydaline in the presence of an NADPH-generating system resulted in the formation of nine metabolites, namely, four *O*-desmethylcorydaline [M1 (yuanhunine), M2 (9-*O*-desmethylcorydaline), M3 (isocorybulbine), and M4 (corybulbine)], three di-*O*-desmethylcorydaline [M5 (9,10-di-*O*-desmethylcorydaline), M6 (2,10-di-*O*-desmethylcorydaline), and M7 (3,10-di-*O*-desmethylcorydaline)], M8 (hydroxyyuanhunine), and M9 (hydroxycorydaline). Incubation of corydaline in human hepatocytes produced four metabolites including M1, M5, M6, and M9. *O*-Demethylation and hydroxylation were the major metabolic pathways for the metabolism of corydaline in human liver microsomes and hepatocytes.

Keywords: Corydaline metabolism / Human hepatocytes / Human liver microsomes / LC-MS^n
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1 Introduction

Corydalis tuber, the root of *Corydalis yanhusuo* W.T. Wang, has long been used as a herbal drug due to its analgesic and antiulcer effects [1–3]. Corydaline, 2,3,9,10-tetramethoxy-13-methyl-5,8,13,13a-tetrahydro-6H-isoquino[3,2-a]isoquinoline, is isolated from Corydalis tuber and has been demonstrated to show acetylcholinesterase and butylcholinesterase inhibitory activity [4–6], antiallergic activity [7], and antinociceptive activity [8], and to facilitate gastric accommodation [9].

As for CYP450 aspects, corydaline was found to potently inhibit CYP2C19-catalyzed *S*-mephenytoin 4′-hydroxylatoin and CYP2C9-catalyzed diclofenac 4-hydroxylation with K_i values of 1.7 and 7.0 μM, respectively, in human liver microsomes [10]. Corydaline was shown to be a mechanism-based CYP3A4 inhibitor with the rate of enzyme inactivation (k_{inact}) and K_i value of 0.064 min^{-1} and 30.0 μM, respectively [10], suggesting that corydaline may be metabolized in human liver microsomes.

To the best of our knowledge, no previous studies have been reported on the metabolism of corydaline in animals or humans, although the metabolism of corydaline is important in its pharmacodynamics and toxicity. This study aimed to identify the metabolites of corydaline formed from *in vitro* incubation of corydaline with human liver microsomes and hepatocytes by using liquid chromatography-ion trap mass spectrometry (LC-MS^n).

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Abbreviations: CYP, cytochrome P450; K_i, inhibition constant; **NADP$^+$**, nicotinamide adenine dinucleotide phosphate; **NADPH**, reduced nicotinamide adenine dinucleotide phosphate

2 Materials and methods

2.1 Materials

Corydaline (>98%) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corybulbine, isocorybulbine, and yuanhunine were isolated from the tubers of *Corydalis turtschaninovii* using our previous method [11]. Nicotinamide adenine dinucleotide phosphate (NADP$^+$), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pooled human liver microsomes (H161), human cryopreserved hepatocytes (donor no. HH227), and cryopreserved hepatocyte purification kit were obtained from BD Gentest Co (Woburn, MA, USA). Methanol and methyl *tert*-butyl ether (HPLC grade) were obtained from

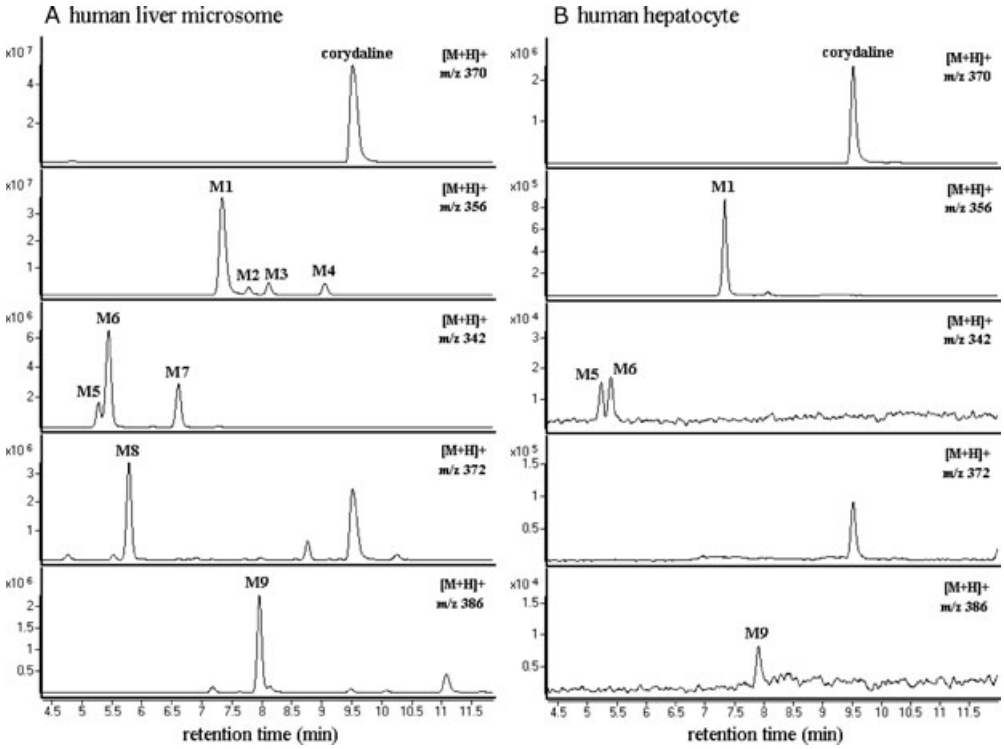

Figure 1. Extracted ion chromatograms of corydaline and its metabolites obtained after incubation of corydaline with (A) human liver microsomes in the presence of NADPH-generating system for 1 h and (B) human hepatocytes at 37°C for 2 h.

Burdick and Jackson Inc. (Muskegon, MI, USA), and the other chemicals were of the highest quality available.

2.2 *In vitro* metabolism of corydaline and related compounds in human liver microsomes

The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 3 mM magnesium chloride, pooled human liver microsomes (250 μg protein), reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.5 U glucose-6-phosphate dehydrogenase), and 10 μM of corydaline in a total volume of 250 μL. To help the confirmation of the metabolites from corydaline, the authentic corybulbine, isocorybulbine, and yuanhunine were also incubated as described above. Control incubations were conducted in the absence of NADPH-generating system. The reaction mixtures were incubated at 37°C for 60 min, and the reaction was stopped by adding 1000 μL of methyl *tert*-butyl ether. The reaction mixture was vortex mixed and centrifuged at 10 000 × *g* for 5 min, and 900 μL of the organic layer was evaporated to the dryness at 35°C for 10 min using a vacuum concentrator (EZ-2 plus, Genevac, Ipswich, UK). The residue was dissolved in 50 μL of 20% methanol and an aliquot (5 μL) was analyzed by $LC\text{-}MS^n$ to identify the metabolites.

2.3 *In vitro* metabolism of corydaline in human hepatocytes

Cryopreserved human hepatocytes were recovered with a cryopreserved hepatocyte purification kit and the viable hepatocytes were resuspended in William E buffer at a final concentration of 2×10^6 cells/mL. Hundred microliter of human hepatocyte suspensions (2×10^5 cells) and 100 μL of 10 μM corydaline were added to 96 well plate and the mixtures were incubated for 120 min at 37°C in CO_2 incubator. One hundred and fifty microliter of the incubation mixture was transferred to 1.5 mL eppendorf tube and 1000 μL of methyl *tert*-butyl ether was added and vortex mixed. The mixture was centrifuged at 10 000 × *g* for 5 min and 900 μL of the organic layer was evaporated to the dryness at 35°C for 10 min using a vacuum concentrator. The residues were dissolved in 40 μL of 20% methanol by vortex mixing for 2 min. The aliquot (5 μL) was injected onto a $LC\text{-}MS^n$ system.

2.4 $LC\text{-}MS^n$ analysis of corydaline and its metabolites

To separate and identify the structures of corydaline and its metabolites, a Varian 500-MS ion trap mass spectrometer (Walnut Creek, CA, USA) coupled with the Varian 212-LC system was used.

For the separation of corydaline and its metabolites, the various columns such as Kinetex PFP column (2.6 μm, 2.1 mm i.d. × 50 mm, Phenomenex, Torrance, CA, USA), Kinetex C18 column (2.6 μm, 2.1 mm i.d. × 50 mm, Phenomenex), Pinnacle DB biphenyl column (1.9 μm, 2.1 mm i.d. × 50 mm, Restek Co., Bellefonte, PA, USA), and Chromolith® Performance RP-18e column (2 μm, 3.0 mm i.d. × 100 mm, Merck KGaA, Darmstadt, Germany) were tried using methanol and 0.1% formic acid as mobile

Table 1. Product ions of corydaline and its nine metabolites obtained by LC-MS^n analysis

Compounds	Retention time (min)	$[M+H]^+$ (*m/z*)	Product ion tree (*m/z*) ($MS^1>MS^2>MS^3>MS^4>MS^5$)
Corydaline	9.6	370	370 >192 >177 >148 >121; 370 >192 >177 >146 >118; 370 >192 >176 >146 >118; 370 >165 >150 >135 >107
M1 (Yuanhunine)	7.2	356	356 >192 >177 >148 >121; 356 >192 >177 >146 >118 356 >165 >150 >135 >107, 105
M2 (9-*O*-Desmethyl-corydaline)	7.7	356	356 >192 >151; 356 >178 >163 >135 >107, 106
M3 (Isocorybulbine)	8.1	356	356 >178 >163 >135 >107, 106; 356 >192 >151
M4 (Corybulbine)	9.0	356	356 >192 >177 >148 >121; 356 >165 >150 >135 >107
M5 (9,10-di-*O*-Desmethylcorydaline)	5.2	342	342 >327 >312 >280 >208; 342 >327 >178 >163 >135; 342 >327 >151
M6 (2,10-di-*O*-Desmethylcorydaline)	5.4	342	342 >327 >312 >280 >208; 342 >327 >178 >163 >135; 342 >327 >151: 342 >190
M7 (3,10-di-*O*-Desmethylcorydaline)	6.6	342	342 >165 >150 >135 >107, 105; 342 >192 >176
M8 (Hydroxyyuanhunine)	5.8	372	372 >192 >177 >146 >118; 372 >192 >177 >148
M9 (Hydroxycorydaline)	7.9	386	386 >368 >336 >320 >305, 277; 386 >368 >177

phase. Best separation of metabolites was obtained with a Chromolith® Performance RP-18e column using a gradient elution of 5% methanol in 0.1% formic acid (mobile phase A) and 95% methanol in 0.1% formic acid (mobile phase B) at a flow rate of 0.8 mL/min: 5% mobile phase B from 0 min to 0.5 min, 5% to 25% mobile phase B from 0.5 min to 8 min, 25% mobile phase B from 8.0 min to 11.5 min, 25% to 5% mobile phase B from 11.5 min to 11.6 min, and 5% mobile phase B from 11.6 min to 16 min.

MS^n spectra for corydaline and its metabolites were recorded by electrospray ionization in the positive mode, and the electrospray source settings were as follows: needle voltage, 5000 V; drying gas temperature, 350°C; nebulizer gas pressure, 50 psi; drying gas pressure, 30 psi; capillary voltage, 63 V. Turbo Data Dependent Scanning (TurboDDS™) (Varian) was used to obtain MS/MS spectra up to MS^5. The proposed structures for the product ions of corydaline and its metabolites were determined by using Mass Frontier software (version 6.0; HighChem Ltd., Slovakia).

3 Results and discussion

LC-MS analysis of the organic extracts obtained after human liver microsomal incubation of corydaline in the presence of NADPH-generating system resulted in nine metabolites (M1–M9) with unchanged corydaline (Fig. 1A). The protonated molecular ions ($[M+H]^+$) for corydaline and its nine metabolites including M1, M2, M3, M4, M5, M6, M7, M8, and M9 were observed at *m/z* 370, 356, 356, 356, 356, 342, 342, 342, 372, and 386, respectively. M1, M2, M3, M4, M5, M6, M7, M8, M9, and corydaline represented approximately 32.7, 1.5, 2.8, 2.7, 0.6, 3.9, 1.9, 2.1, 1.3, and 50.3% of the total integrated peak areas, respectively, after 1 h incubation period. The incubation of corydaline with human hepatocytes resulted in four metabolites of M1, M5, M6, and M9 (Fig. 1B).

Chromatographic separation of metabolites was needed to unambiguously identify the structure of the metabolites because M1, M2, M3, and M4 have the same $[M+H]^+$ ions of *m/z* 356, while M5, M6, and M7 have the same $[M+H]^+$ ions at *m/z* 342. After many trials with different columns and mobile phase combinations, good resolution of corydaline and its nine metabolites was established on a Chromolith® Performance RP-18e column using the gradient elution of methanol and 0.1% formic acid compared to Kinetex PFP column, Kinetex C18 column, and Pinnacle DB biphenyl column (Fig. 1, Table 1).

Ion tree reports from MS^5 analysis of unchanged corydaline and its nine metabolites produced the prominent and informative product ions for structural elucidation (Fig 2. and Table 1). MS^2 spectra of corydaline and its nine metabolites are shown in Fig. 3.

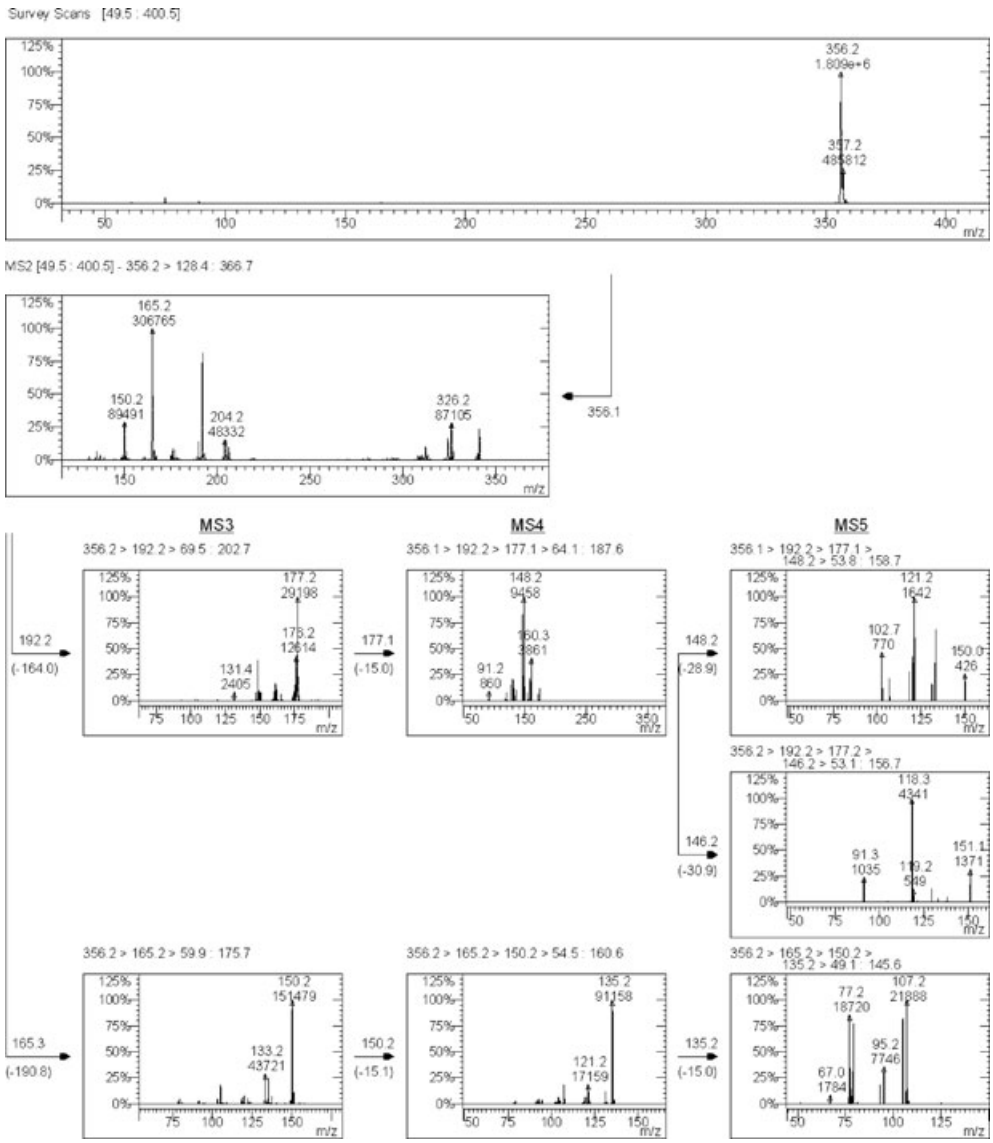

Figure 2. Ion tree report for MS^5 fragmentation of M1 (yuanhunine).

MS^n spectra of corydaline yielding a $[M+H]^+$ ion at *m/z* 370 showed the characteristic product ions at *m/z* 192 and 165 for MS^2, *m/z* 177, 176, and 150 for MS^3, *m/z* 148, 146, and 135 for MS^4, *m/z* 121, 118, and 107 for MS^5 (Table 1). The ion chemistry for these product ions of corydaline was shown in Fig. 4A.

M1, M2, M3, and M4 showed $[M+H]^+$ ion at *m/z* 356, which is 14 amu less than $[M+H]^+$ ion of corydaline in the mass spectra, suggesting that those metabolites were formed from corydaline via *O*-demethylation. By comparison with the retention times and MS^n spectra of corresponding authentic standards, M1, M3, and M4 were identified as yuanhunine, isocorybulbine, and corybulbine, respectively (Figs. 4B, 5B, and 5C). M1 (yuanhunine) was identified as a major metabolite of corydaline in human hepatocytes as well as human liver microsomes. MS^n spectra of M2 showed the product ions at *m/z* 192 and 178 for MS^2, *m/z* 163 and 151 for MS^3, *m/z* 135 for MS^4, and *m/z* 107 for MS^5, suggesting that *O*-demethylation might have occurred at 9-methoxy moiety of corydaline (Table 1, Fig. 5A). M2 was tentatively identified as 9-*O*-desmethylcorydaline.

All of M5, M6, and M7 showed $[M+H]^+$ ion at *m/z* 342, which is 28 amu less than $[M+H]^+$ ion of corydaline, corresponding to the loss of two methyl groups from corydaline (Table 1). M5, M6, and M7 were also identified after human liver microsomal incubation of yuanhunine in the presence of NADPH, indicating that M5, M6, and M7 might be di-*O*-desmethylcorydaline formed from M1 (yuanhunine) *via* *O*-demethylation at 9-, 2-, and 3-methoxy moiety, respectively.

MS^n spectra of M5 produced the characteristic product ions at *m/z* 327 and 192 for MS^2, *m/z* 312, 178, and 151 for MS^3, *m/z* 280 and 163 for MS^4, *m/z* 208 and 135 for MS^5, suggesting that the loss of two methyl groups might have

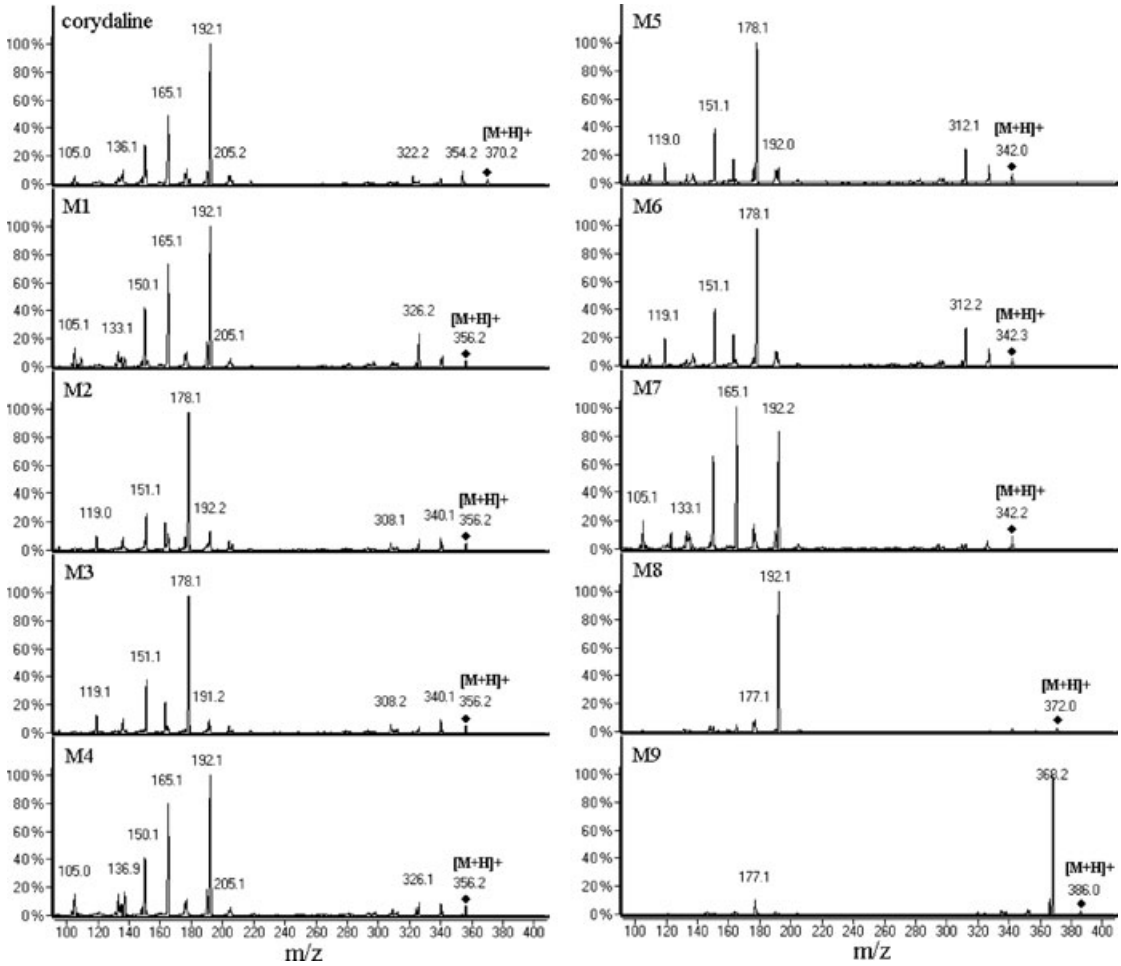

Figure 3. MS^2 spectra of corydaline and its nine metabolites (M1–M9) obtained by LC-MS^n analysis of the human liver microsomal incubates of corydaline in the presence of an NADPH-generating system.

occurred at both 9- and 10-methoxy moieties from corydaline (Fig. 6A) . M5 was produced from yuanhunine but was not formed from isocorybulbine and corybulbine, supporting that M5 might be 9,10-di-*O*-desmethylcorydaline.

M6 was produced after human liver microsomal incubation of isocorybulbine as well as yuanhunine in the presence of NADPH, suggesting that M6 could be 2,10-di-*O*-desmethylcorydaline. MS^n spectra of M6 produced the characteristic product ions at *m/z* 327 and 190 for MS^2, *m/z* 312, 178, and 151 for MS^3, *m/z* 280 and 163 for MS^4, and *m/z* 208 and 135 for MS^5, supporting that the loss of two methyl groups might have occurred at both 2- and 10-methoxy moieties from corydaline (Fig. 6B).

M7 was identified after human microsomal incubation of corybulbine and yuanhunine in the presence of NADPH, indicating that the loss of two methyl groups could be occurred at both 3- and 10-methoxy moieties from corydaline. From MS^n spectra of M7, the characteristic product ions were observed at *m/z* 192 and 165 for MS^2, *m/z* 176 and 150 for MS^3, *m/z* 135 for MS^4, and *m/z* 107 for MS^5, supporting that M7 might be 3,10-di-*O*-desmethylcorydaline (Fig. 7A).

M8 was produced after human microsomal incubation of yuanhunine as well as corydaline in the presence of NADPH. However, M8 was not formed from corybulbine and isocorybulbine. M8 produced an $[M+H]^+$ ion at *m/z* 372, which is 2 amu higher than $[M+H]^+$ ion of corydaline and 16 amu higher than M1 (yuanhunine), indicating 10-*O*-demethylation and hydroxylation of corydaline. MS^n spectra of M8 generated the characteristic product ions at *m/z* 192 for MS^2, *m/z* 177 for MS^3, *m/z* 148 and 146 for MS^4, and *m/z* 118 for MS^5 (Fig. 7B). From these results, M8 was tentatively identified as hydroxyyuanhunine but the position of hydroxylation was not accurately identified.

M9 produced an $[M+H]^+$ ion at *m/z* 386, which is 16 amu higher than $[M+H]^+$ ion of corydaline, suggesting hydroxylation of corydaline. M9 generated the characteristic product ions at *m/z* 368 (a loss of H_2O from $[M+H]^+$) for MS^2, *m/z* 336 and 177 for MS^3, *m/z* 320 for MS^4, and *m/z* 305 and 277 for MS^5 (Fig. 7C). M9 was tentatively characterized as hydroxycorydaline, but the exact site for hydroxylation could not be determined.

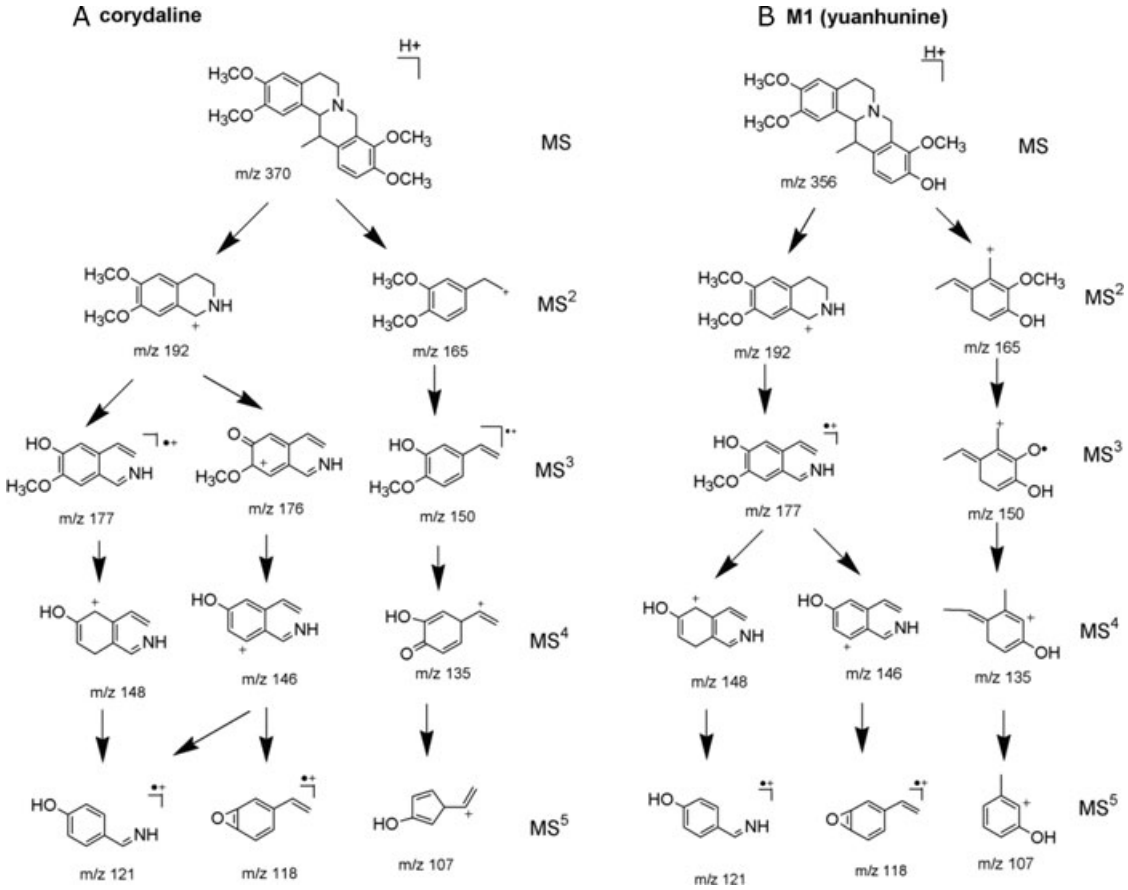

Figure 4. MS^n fragmentation schemes for (A) corydaline and (B) M1 (yuanhunine).

On the basis of these results, the possible metabolic pathways of corydaline in human liver microsomes and hepatocytes are shown in Fig. 8. Corydaline was metabolized to M9 (hydroxycorydaline) and four *O*-desmethylcorydaline such as M1 (yuanhunine), M2 (9-*O*-desmethylcorydaline), M3 (isocorybulbine), and M4 (corybulbine), which were further metabolized to M5 (9,10-di-*O*-desmethylcorydaline), M6 (2,10-di-*O*-desmethylcorydaline), M7 (3,10-di-*O*-desmethylcorydaline), and M8 (hydroxyyuanhunine).

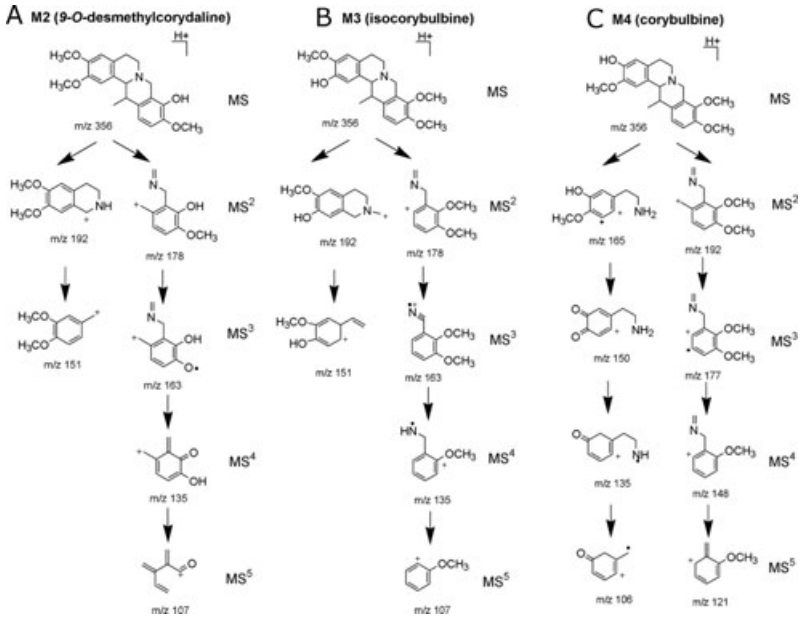

Figure 5. MS^n fragmentation schemes for (A) M2 (9-*O*-desmethylcorydaline), (B) M3 (isocorybulbine), and (C) M4 (corybulbine).

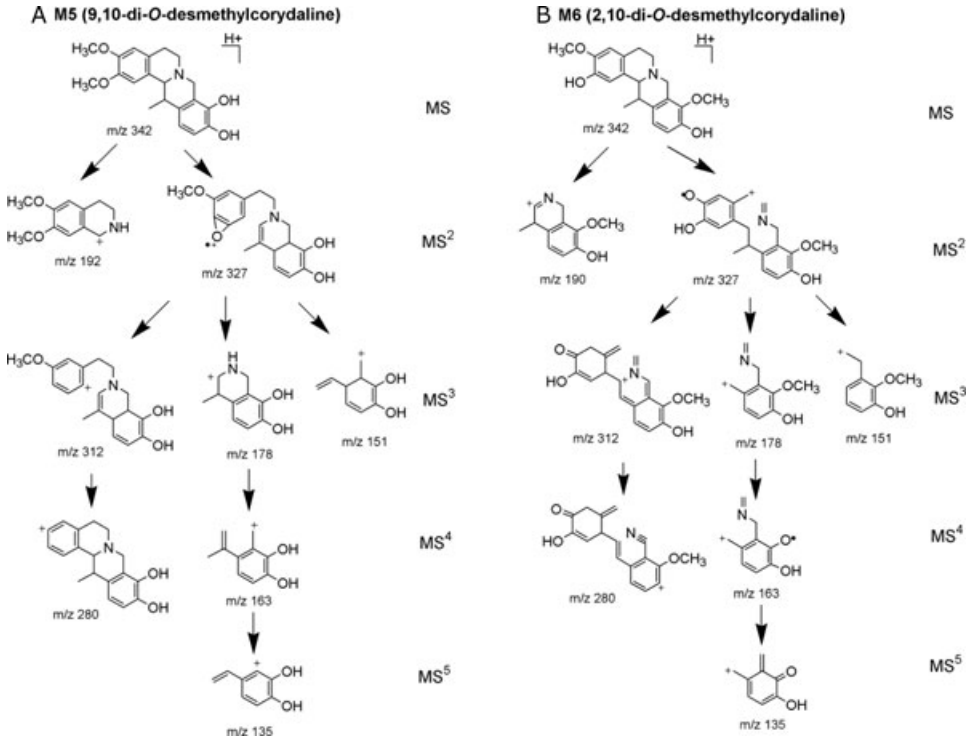

Figure 6. MS^n fragmentation schemes for (A) M5 (9,10-di-*O*-desmethylcorydaline) and (B) M6 (2,10-di-*O*-desmethylcorydaline).

4 Concluding remarks

The metabolism of corydaline human liver microsomes and hepatocytes was for the first time elucidated in the present study. Corydaline was metabolized to nine metabolites in human liver microsomes by means of *O*-demethylation and hydroxylation: M1 (yuanhunine), M2 (9-*O*-desmethylcorydaline), M3 (isocorybulbine), M4 (corybulbine), M5 (9,10-di-*O*-desmethylcorydaline), M6 (2,10-di-*O*-desmethylcorydaline), M7 (3,10-di-*O*-desmethylcorydaline),

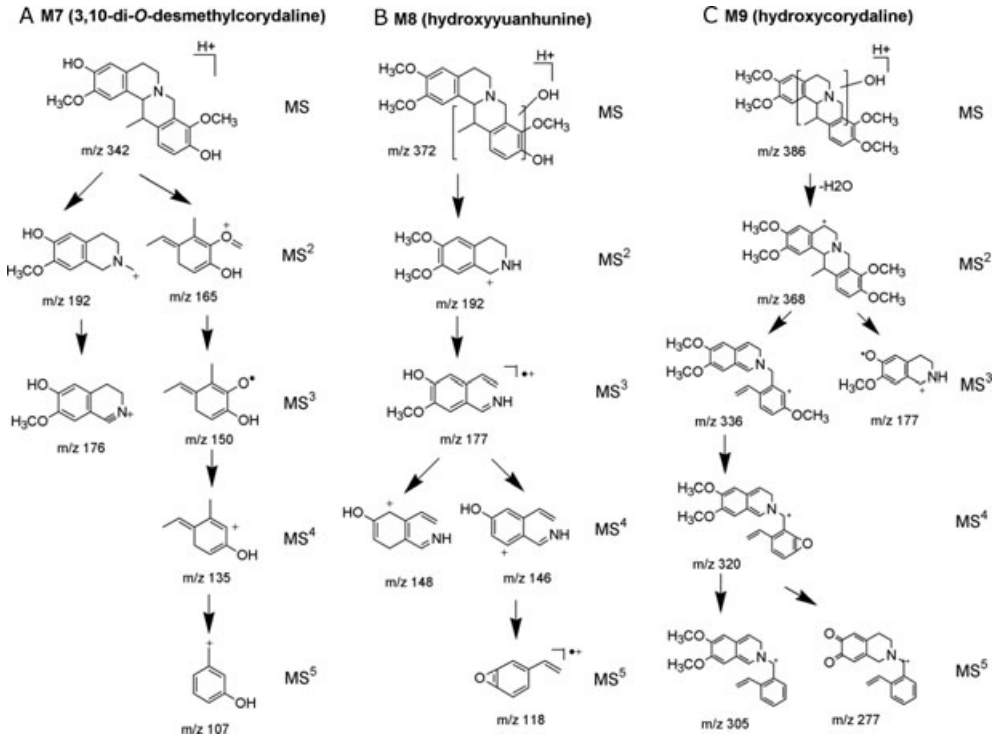

Figure 7. MS^n fragmentation schemes for (A) M7 (3,10-di-*O*-desmethylcorydaline), (B) M8 (hydroxyyuanhunine), and (c) M9 (hydroxycorydaline).

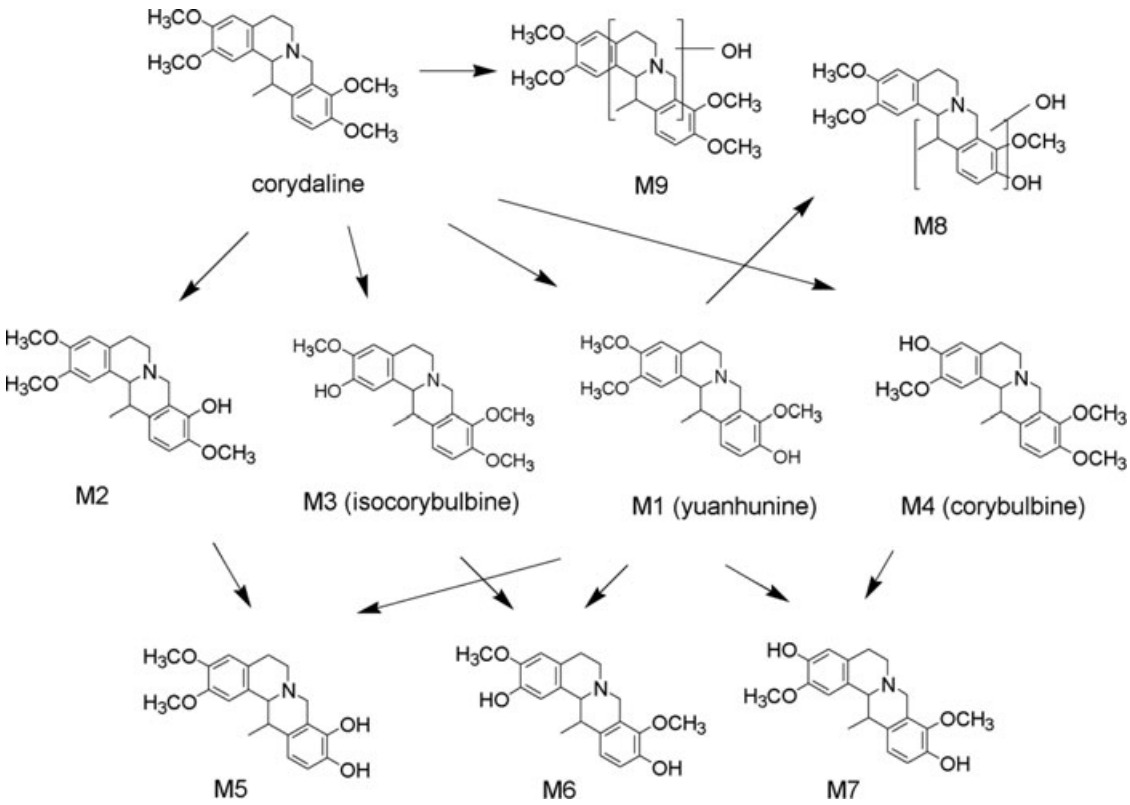

Figure 8. Possible metabolic pathways of corydaline in human liver microsomes.

M8 (hydroxyyuanhunine), and M9 (hydroxycorydaline). M1, M5, M6, and M9 were also identified after incubation of corydaline in human hepatocytes.

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The authors have declared no conflict of interest.

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