Metabolism of Dicentrine: Identification of the Phase I and Phase II Metabolites in Miniature Pig Urine

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ABSTRACT:
The metabolic profile of dicentrine, a selective α1-adrenoceptor antagonist with potent antiarrhythmic and antihypertensive activities, in miniature pig urine via oral administration was investigated for the first time. The urine, collected after a single oral administration of dicentrine, was pretreated using solvent extraction and column chromatographic methods to identify the metabolites containing fractions. Twenty-four metabolites (MI-1–9 and MII-1–15), of which 21 compounds are new, were identified by mass spectrometry and high-performance liquid chromatography–diode array detector solid-phase extraction–NMR techniques. Of these, 14 metabolites (MI-5, MII-1 and 2, and MII-5-15) were further isolated for structure confirmation. The phase I metabolic transformations of dicentrine were found to be N-demethylation, N-oxidation, O-demethylation (9,10-OMe), O,O-demethylation (1-OCH2O2), and hydroxylation at the benzylic (C-4) and the aromatic (C-3) positions, whereas those for the phase II were O-glucuronidation and O-glucosylation of the phenolic group of the phase I metabolites.

Introduction
(+)-Dicentrine, an aporphine alkaloid present abundantly in a variety of genera in the plant families Lauraceae (Chen et al., 1991), Papaveraceae (Lalezarì et al., 1976), Menispermaceae (De Wet et al., 2007), and Fumariaceae (Istailov et al., 1984), was demonstrated to be a selective α1-adrenoceptor antagonist (Teng et al., 1991) and to possess potent biological activities, including antiarrhythmic (Su et al., 1994), antiplatelet aggregation (Teng et al., 1991; Yu et al., 1992a), antihypertensive (Yu et al., 1992b), antihyperlipidemic (Yu et al., 1994), antiplatelet aggregation (Teng et al., 1991; Yu et al., 1994), and antitumor (Stévigny et al., 2005) activities. The antiarrhythmic effect of dicentrine has been shown in rabbit (Young et al., 1994) and dog (Chang et al., 1995) models. These results showed that dicentrine was a promising drug candidate as a class I and class III antiarrhythmic agent. Another study revealed that dicentrine could relieve bladder outlet obstruction caused by benign prostatic hyperplasia (Yu et al., 1994). Oral administration of dicentrine (5 mg/kg) to conscious spontaneously hypertensive rats resulted in a significant reduction in mean arterial pressure, which was observed 1 h after administration, and the hypotensive effect persisted for more than 15 h (Yu et al., 1992b). This long-acting phenomenon indicated that dicentrine had either a long half-life or active metabolites. One pharmacokinetic study of dicentrine (10 mg/kg i.v.) in rat (Tsai et al., 1996) revealed that the distribution and elimination of dicentrine were extremely fast [t1/2; 4.32 min, t1/2B; 45.20 min, and clearance 0.51 l/(kg · min)], indicating that dicentrine was easily metabolized. Thus, the long-acting effect should arise from the active metabolites. However, dicentrine metabolites have not been reported yet. Therefore, we investigated the metabolites in the urine of miniature pigs after intragastric oral administration of dicentrine mesylate.

Because the amounts of metabolites are usually minute, their structures are commonly analyzed by liquid chromatography–MS/MS. However, the information provided by this technique is usually not enough to determine the exact structure. Up to now, NMR and HPLC were recognized generally to be the most powerful tools for structure elucidation and analytic scale separation, respectively. Hence, the HPLC-NMR technique has been applied to study the metabolic profile of some drugs (Burton et al., 1997; Spraul et al., 2003). The HPLC-SPE-NMR technique has also been successfully applied in analysis of natural products (Wang and Lee, 2005; Lam et al., 2007; Lee et al., 2007) as well as in identification of certain metabolites (Godejohann et al., 2004; Sandvoss et al., 2005). In the present study, HPLC-SPE-NMR, along with HPLC-HRESIMS, was applied to the characterization of the dicentrine metabolites in the urine of miniature pigs. The structures of major metabolites were confirmed by spectral analysis through further isolation.

ABBREVIATIONS: MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; HRESIMS, high-resolution electrospray ionization mass spectrometry; MeOH, methanol; MS, mass spectrometry; 2D, two-dimensional; HMOC, heteronuclear multiple quantum correlation; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; DAD, diode array detector; RP, reverse-phase; TFA, trifluoroacetic acid; ESI, electrospray ionization; 1D, one-dimensional; GlcUA, glucuronyl.
Materials and Methods

Materials and Supplies. HPLC-pure dicentrine (1) was provided by Dr. Chien-Chih Chen, National Research Institute of Chinese Medicine, Taipei, Republic of China. Its mesylate salt, m.p. 262.1°C (HPLC-pure) was prepared by recrystallization of the mixture of dicentrine and methanesulfonic acid in equal amounts from MeOH. An analytical HPLC column (Prodigy ODS3 100A, 250 × 4.6 mm, 5 µm; Phenomenex, Torrance, CA) was used in HPLC-SPE-NMR and HPLC-MS. A semipreparative HPLC column (Prodigy ODS3 100A, 250 × 10 mm, 5 µm; Phenomenex) was used for the isolation of metabolites. For the isolated compounds, the 1H NMR and 2D NMR (HMQC or HSQC, HMBc, or NOESY) spectra were measured in an inverse probe of a 600-MHz Avance III NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), and the 13C NMR spectra were measured either in a ZNP probe of a 400-MHz Avance III spectrometer. MS data were measured by an Esquire 2000 ion trap mass spectrometer (Bruker Daltonics BioSpin) and a microTOF II time-of-flight mass spectrometer with electrospray ion sources (Bruker Daltonics).

Laboratory Animals. Two mature (6 months old) female Langy small-eared miniature pigs were bred at Taitung Breeding Animal Propagation, Taiwan. Livestock Research Institute, Taitung, Taiwan and were transported to the National Taiwan University Veterinary Hospital for the experiment. They were housed in a sheltered outdoor fenced space with good ventilation and allowed forage and water ad libitum throughout the experiment. The animal experiment was conducted according to a protocol approved by the Institutional Animal Care and Use Committee of National Taiwan University [Approval 95 (073)].

Animal Dosing. Two miniature pigs with the respective weights of 18 and 20 kg were starved 15 h before feeding of dicentrine mesylate (51.3 mg/kg, equivalent to 40.0 mg of dicentrine/kg) through a soft plastic gastric tube. Then the pigs were kept in metabolic cages equipped with normal fodder and water. The urine and feces were collected separately and were stored at 4°C before further processing.

Urine Collection and Pretreatment Procedures. The urine of the first pig was collected over a 0- to 24-h period to give approximately 500 ml, which was passed through an Amberlite XAD-2 column and eluted with H2O (1.5 liters, fraction A-I) first, followed by MeOH (1.5 liters). The MeOH eluant was evaporated at reduced pressure at 40°C to give the MeOH fraction (370 mg, fraction A-II). Fractionation of fraction A-II on a Sephadex LH-20 column (MeOH-H2O, 7:3) gave three metabolites containing fractions (fraction A-II-1, 7.8 mg; fraction A-II-2, 16.9 mg; and fraction A-II-3, 9.0 mg, monitored by DAD).

The urine of the second pig was collected separately at intervals of 0 to 14.0, 14.0 to 26.0, and 26.0 to 48.0 h. These urine samples were freeze-dried first and then were extracted by 90% MeOH (two 20-ml extractions). The extracts were evaporated at reduced pressure at 40°C and then were analyzed by HPLC-DAD-MS. The 90% MeOH, urine extract collected at 14 to 26 h (fraction B-I, 9.86 g) was fractionated on a Sephadex LH-20 column (MeOH-H2O, 8:2, o.d. 3.5 cm, height 62 cm) to give a metabolite-containing fraction (fraction B-I-1, 1.26 g).

Feaces Collection and Pretreatment Procedures. The feces samples were collected over a 0- to 48-h period. They were suspended in 90% MeOH (5 ml) in a 15-ml centrifuge tube, vortexed, and centrifuged for 10 min at 3000 rpm. The supernatant was collected. This procedure was repeated three times. The combined supernatants were evaporated under reduced pressure at 40°C to yield a residue (560 mg, fraction C). A portion of this residue (10 mg) was dissolved in MeOH (1.0 ml) and filtered through a 0.45-µm membrane for HPLC-DAD-MS analysis.

Materials and Methods

Isolation of MII-1 and 2 and -5–15 from Fraction B-I-1. Fraction B-I-1 (24.4 mg, 15 times; 366 mg in 1.2 ml of H2O) was chromatographed on a semipreparative RP-HPLC column delivered by linear gradient of MeCN-0.1% TFAaq 11:89, to give a metabolite-containing fraction (24.4 mg, 15 times; 366 mg in 1.2 ml of H2O) was chromatographed on a semipreparative RP-HPLC column delivered by linear gradient of MeCN-0.1% TFAaq 11:89, to give a metabolite-containing fraction (fraction B-I-1, 2.5 mg (20 µl of MeOH), three times; fraction A-II-1.1.53 mg (20 µl of MeOH), three times; and fraction A-II-3 1.57 mg (20 µl of MeOH), three times).

HPLC-HRESIMS and MS/MS Analysis. An HPLC-ESI ion trap mass spectrometer (Esquire 2000) was used in the beginning to trace the dicentrine metabolites in each HPLC fraction. A small portion (5%) of the HPLC flow (HPLC flow rate 0.5 ml/min) was directed into the mass spectrometer via a splitter (1:20). The temperature at the ESI interface heated capillary was set at 357 K, electrospray voltage was set to 4.5 kV, the nebulizer gas (nitrogen) pressure was set to 23 psi, and a dry nitrogen flow of 8.0 l/min was used. The trap drive was set to 29 to 37 (arbitrary units). The capillary exit voltage was set to 101 to 110 V for the positive ion mode and to −101 to 110 V for the negative ion mode. A specific collision energy (0.5 V) was chosen at each fragmentation step for all of the compounds investigated. The function of smart fragmentation was on (SmartFrag Ampl was 30–200%), and the isolation width was 4 m/z. Low-resolution electrospray ionization mass spectrometry and MS/MS data were acquired in both positive and negative modes over a scan range of m/z 50 to 1000.

HRESIMS Analysis. Time-of-flight mass spectrometry (microTOF II) was used to measure the accurate molecular weight of each metabolite. Both HPLC-HRESIMS and direct infusion of pure compounds were performed. The spectrometer was operated under the following conditions: ESI in positive/ negative mode and a mass range of m/z 50 to 1000. The ESI source was set to the following conditions: drying gas flow rate 4.0 l/min, drying gas temperature 180°C, nebulizer 0.4 bar, and capillary voltage 4.1 kV.

Isolation of MI-1 and 2 and -5–15 from Fraction B-I-1. Fraction B-I-1 (24.4 mg, 15 times; 366 mg in 1.2 ml of H2O) was chromatographed on a semipreparative RP-HPLC column delivered by linear gradient of MeCN-0.1% TFAaq 11:89, to give a metabolite-containing fraction (fraction B-I-1, 2.5 mg (20 µl of MeOH), three times; fraction A-II-1.1.53 mg (20 µl of MeOH), three times; and fraction A-II-3 1.57 mg (20 µl of MeOH), three times).

Feaces Collection and Pretreatment Procedures. The feces samples were collected over a 0- to 48-h period. They were suspended in 90% MeOH (5 ml) in a 15-ml centrifuge tube, vortexed, and centrifuged for 10 min at 3000 rpm. The supernatant was collected. This procedure was repeated three times. The combined supernatants were evaporated under reduced pressure at 40°C to yield a residue (560 mg, fraction C). A portion of this residue (10 mg) was dissolved in MeOH (1.0 ml) and filtered through a 0.45-µm membrane for HPLC-DAD-MS analysis.

Results

HPLC Analysis of the Phase I Metabolites (MI-1–9). The urine collected from the first miniature pig in 24 h after oral administration of the mesylate salt of dicentrine (I) (40 mg/kg) was pretreated and separated into metabolite-containing fractions (fraction A-II and subfractions). RP-HPLC and HPLC-MS analyses of these fractions, using the delivery system MeCN-0.1% TFAaq as indicated under Materials and Methods, led to the finding of nine phase I metabolites (MI-1–9) (Fig. 1) and the parent compound (1) as their TFA salts, for which compound labeling was done according to the elution order observed in the RP-HPLC chromatograms. Compounds characterized from fraction A-II were MI-4, tR 35.51 min; MI-5, tR 36.47 min; MI-8, tR 42.96 min. Results

HPLC Analysis of the Phase I Metabolites (MI-1–9). The urine collected from the first miniature pig in 24 h after oral administration of the mesylate salt of dicentrine (1) (40 mg/kg) was pretreated and separated into metabolite-containing fractions (fraction A-II and subfractions). RP-HPLC and HPLC-MS analyses of these fractions, using the delivery system MeCN-0.1% TFAaq as indicated under Materials and Methods, led to the finding of nine phase I metabolites (MI-1–9) (Fig. 1) and the parent compound (1) as their TFA salts, for which compound labeling was done according to the elution order observed in the RP-HPLC chromatograms. Compounds characterized from fraction A-II were MI-4, tR 35.51 min; MI-5, tR 36.47 min; MI-8, tR 42.96 min.
HPLC-SPE-NMR Analysis of the Phase I Metabolites. Dicen-
trine (I) showed characteristic signals for the aromatic H-3, H-8, and
H-11 and 1-OC3H7O-2 and the aliphatic H-6a and N-Me (Table 1;
Supplemental Fig. S1). However, the latter two signals are shifted
downfield if the TFA salt is formed (Lee et al., 2007).

Metabolites MI-4, -8, and -9 were characterized as the known
lastourvilline (O.O-demethylerythrodiicentrene (Eloymi-Ropivia et
al., 1985), actinodaphnine (N.O'-dimethylerythrodiicentrene (Lee and Yang,
1992), and cassythicine (N-methylactinodaphnine, O'-demethyl-
dicentrene) (Tewari et al., 1972; Hara et al., 1986), respectively, based on
the online 1H NMR (Supplemental Fig. S1 and Table S1) and the
HRESIMS analyses.

Metabolites MI-1-3 and -5-7 are all 4β-oxogenated diicentrene
derivatives, as exemplified by their 1H NMR spectra (Table 1; Sup-
plemental Fig. S1), all showing a downfield shifted broad singlet with δ > 4.30 ppm, as observed for the Hα-4 in (4R,6αS)-4-hydroxy-
aphorphines (Hoshino et al., 1975; Yang et al., 1993). The Hα-4 in
(4S,6αS)-4-hydroxyaporphines would appear as a double doublet (J =
6.2, 9.5 Hz) (Hartenstein and Satzinger, 1977). Metabolite MI-5 had
the molecular formula C19H19NO5, as deduced from HRESIMS showing
[M + H]+ at m/z 344.1451 (calc. 344.1498), with two oxygen atoms
more than cassythicine (MI-9), suggesting that MI-5 is
4α-hydroxycassycythicine. The NOESY spectrum of MI-5 showing the
NOE relationship between H-3 (δ 6.86) and H-4 (δ 4.68, br s), and
between H-11 (δ 7.70) and 10-OME (δ 3.87) (Supplemental Fig. S4)
supported this structure elucidation. Metabolite MI-7 had the molec-
ular formula C20H21NO5, as deduced from HRESIMS showing
[M + H]+ at m/z 356.1484 (calc. for C20H21NO5, 356.1498), with
an oxygen atom more than dicentrene (I), suggesting that it was
4α-hydroxydicentrene, a diastereoisomer of the known 4β-hydroxy-
dicentrene (Vecchietti et al., 1979). The 1H NMR spectrum of MI-7.TFA
(Table 1; Supplemental Fig. S1) being almost identical to that of
MI-5.TFA except for the presence of 9-OMe (δ 3.82) supported this
eclucidation. Metabolite MI-2 had the same molecular formula as
MI-5, as deduced from HRESIMS. Its 1H NMR spectrum was almost
equal to that of MI-5, except for a certain shift for H-8 (δ 6.93 versus δ 6.81, MI-5) and H-11 (δ 7.58 versus δ 7.70, MI-5)
(Table 1; Supplemental Fig. S1). Therefore, MI-2 was reasonably
eclucidated as 4α-hydroxyphanostenine, a structural isomer of MI-5
with exchanged substitutions at C-9 and C-10. Metabolite MI-1 had
the molecular formula C19H21NO5, as deduced from HRESIMS
showing
[M + H]+ at m/z 344.1541 (calc. 344.1498), with one oxygen atom
more than lastourvilline (MI-4). Its 1H NMR spectrum (Table 1; Sup-
plemental Fig. S1) was similar to that of MI-4, except for the downfield shifted H-4 (δ 5.02, br s). These data thus estab-
lished MI-1 as 4α-hydroxylastourvilline. Metabolite MI-3 had the
molecular formula C20H23NO5, as deduced from HRESIMS showing
[M + H]+ at m/z 358.1662 (calc. 358.1654), a CH2 residue more
than MI-1. Its 1H NMR spectrum was similar to that of MI-1.TFA,
except for the presence of one additional MeO singlet (δ 3.44) and
upfield shifted H-3 and H-4 signals (δs 6.92 versus 6.81 in
MI-1.TFA; δH-4 4.36 versus 5.02 in MI-1.TFA) (Table 1; Supplemental
Fig. S1). The latter shift differences were thus located the methoxy
group at C-4 (Nieto et al., 1976). Accordingly, MI-3 was elucidated
as 4α-methoxylastourvilline. Metabolite MI-6 had the molecular
formula C19H19NO5, as deduced from HRESIMS showing
[M + H]+ at m/z 358.1299 (calc. 358.1291), with one oxygen atom more than
MI-5. The 1H NMR spectrum of MI-6.TFA (CD3OD) was similar to
that of MI-5.TFA except for the downfield shifted signals for H-6a (δ
4.61, dd versus δ 4.05, dd, MI-5.TFA) and N-Me (δ 3.57 versus δ
4.30, MI-5.TFA). The latter two signals (H-6a and N-Me), being close
to those in boldine N[O]oxide (δ 4.57 and 3.65, respectively) (Lee et al.,
2007), thus establishing MI-6 as an N[O]oxide. Accordingly, the
structure of MI-6 was elucidated as 4α-hydroxycassycythine
N[O]oxide (Table 1; Supplemental Fig. S1).

For structure confirmation, MI-5 was further isolated. The unam-
biguous 1H and 13C NMR assignments for MI-5 (Table 1) were made
by 2D NMR spectral analyses (HMOC and HMBC) (Supplemental
Fig. S5).

Fig. 1. Proposed phase I metabolic pathways of dicentrene.
HPLC Analysis of the Phase II Metabolites (MII-1–MII-15). The 90% methanol extract of the urine sample collected at 14.0 to 26.0 h from the second pig was fractionated via Sephadex LH-20 to concentrate the phase II metabolites of dicentrine (1) into subfraction fraction B-I-1. HPLC-DAD and HPLC-MS analyses of this fraction, delivered by MeCN-0.1% TFA, led to the finding of 15 metabolites (MII-1, tR 23.80 min; MII-2, tR 25.53 min; MII-3, tR 29.58 min; MII-4, tR 30.79 min; MII-5, tR 31.37 min; MII-6, tR 31.78 min; MII-7, tR 33.92 min; MII-8, tR 35.14 min; MII-9, tR 35.75 min; MII-10, tR 37.19 min; MII-11, tR 39.84 min; MII-12, tR 42.12 min; MII-13, tR 43.83 min; MII-14, tR 45.46 min; and MII-15, tR 45.84 min) (Figs. 1, 2, and 4).

Structure Elucidation of the Phase II Metabolites (MII-1–15). Following the HPLC analytical conditions for MII-1–15, HPLC-SPE-NMR was performed. The online NMR spectra of these compounds are shown in Supplemental Figs. S2 and S3. To confirm the structures of these more sophisticated phase II conjugated metabolites by more extensive 1D and 2D NMR data, most phase II metabolites (MII-1 and -2 and -5–15) were isolated via semipreparative RP-HPLC, using the delivery system MeCN-0.1% TFA.

Monoglucuronides. Metabolites retaining the 1,2-methylenedioxy group (MII-3 and -9–15). MII-3 and -9–15 retained a 1,2-methylenedioxy group as verified by the characteristic 1H NMR signals, an AB or AX system around δ 6.00 (Table 2, Supplemental Table S2).

9-O-Glucuronides (MII-9, -10, -14, and -15). The designation of the 9-O-GlcUA and 10-OME in these four metabolites was confirmed by NOESY spectral analysis: H-8 (δ 7.09, MII-9; δ 6.98, MII-10; δ 7.08, MII-14; and δ 6.98, MII-15) ↔ 9-O-GlcUA H-1 (δ 5.07, MII-9; δ 5.20, MII-10; δ 5.07, MII-14; and δ 5.16, MII-15); H-11 (δ 7.68, MII-9; δ 7.50, MII-10; δ 7.67, MII-14; and δ 7.52, MII-15) ↔ 10-OME (δ 3.80, MII-9; δ 3.67, MII-10; δ 3.81, MII-14; and δ 3.64, MII-15) (Supplemental Tables S2, S20, S22, S30, and S32). The presence of a 4β-hydroxy group in MII-9 and -10 was supported by their 1H NMR data, all of which showed a broad singlet around δ 4.90 in MII-9 and -10 (Hoshino et al., 1975), and confirmed by the NOESY spectra, showing the correlation of H-3 (δ 6.86, MII-9; δ 6.91, MII-10) to H-4α (δ 4.85, MII-9 and δ 4.92, MII-10). In addition, MII-10 and MII-15 are N-demethylated analogs of MII-9 and MII-14, respectively, based on MS and 1H NMR data. These spectral data thus established them as 4R-hydroxyxaccythicine 9-O-β-d-glucuronide (MII-9), 4R-hydroxyxactinodaphnine 9-O-β-d-glucuronide (MII-10), cassythicine 9-O-β-d-glucuronide (MII-14), and actinodaphne 9-O-β-d-glucuronide (MII-15).

10-O-Glucuronide MII-3. Metabolite MII-3 had the molecular formula C_{25}H_{27}NO_{10} as deduced from HRESIMS, being the same as MII-14 (Table 4). The 1H NMR spectrum of MII-3.TFA (Table 2,
**1H NMR data of TFA salts of dicentrine (1), MI-1-3, and MI-5-7 from HBPC-SPME (40 MHz) analysis and 13C NMR and HMBC data for MI5**

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<th>Position</th>
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<th>J (Hz)</th>
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</table>

**CD2OD** adopted from SPE-NMR was similar to that of MI14-TFA except for the shift difference for H-8 (δ 7.03 versus δ 7.08 in MI14-TFA) and H-11 (δ 7.95 versus δ 6.67 in MI14-TFA) (Supplemental Table S2, CD2OD). Accordingly, MI3 is reasonably elucidated as phanostenine 10-O-β-glucuronic acid, a structural isomer of MI14 with exchanged C-9 and C-10 substituents.

**Metabolites containing a 3-glucuronyloxy group (MI11-13).** The 1H NMR spectrum of MI11 showed a broad singlet at δ 5.24 but was missing the N-Me singlet, suggesting the absence of the N-Me group and the presence of a 4β-OH group as stated above (Hoshino et al., 1975). The NOESY spectrum of MI11 showed the correlation of H-8/9-OMe and H-11/10-OMe. The HMBC spectrum (Supplemental Fig. S24) showed the correlation of C-3 (δ 137.6, s)/GlCUA H-1 (δ 5.31, d, J = 7.6 Hz), and H-4 (δ 5.24). This structural information incorporated with the MS data thus established MI11 as 3-β-glucuronyloxy-4R-hydroxynodicentrine. MI12 had the molecular formula C30H52NO11 as deduced from HRESIMS, with one oxygen glucuronyloxy-4-glucuronyloxy-4R-hydroxynodicentrine. MI13 was the phanostenine 10-O-β-glucuronyloxy-4R-hydroxynodicentrine. This structure was confirmed by the HMBC spectrum (Supplemental Fig. S26), showing the correlation of C-3 (δ 137.3, s)/GlCUA H-1 (δ 5.30, d, J = 7.4 Hz) and H-4s (δ 3.00, m and δ 3.21, dd, J = 5.5, 19.0 Hz). MI13 was an N-methylated analog of MI12, as evidenced from 1H NMR data (TFA salt), exhibiting an additional N-Me at δ 3.17, and MS data, with 14 atomic mass units more than MI12. The assignment of 3-O-GlCUA in MI13 was confirmed by the correlation of C-3 (δ 136.6, s)/GlCUA H-1 (δ 5.29, d, J = 6.4 Hz) and H-4s (δ 3.07, m and δ 3.26, m) in the HMBC spectrum (Supplemental Fig. S28). Thus, MI13 was established as 3-β-glucuronyloxy-4R-hydroxynodicentrine.

**Metabolites retaining 9,10-dimethoxy groups (MI4–8).** Metabolites MI4–8 are 1,2-O0,1-demethylenated monoglucuronides as indicated by the 1H NMR spectra, lacking the 1-OCH2O-2 signals but retaining two methoxy singlets (Table 2; Supplemental Table S2).

**1-O-Glucuronides (MI5 and -8).** The presence of 1-O-GlCUA in MI8 (lastourvilline 1-0-glucuronide) was elucidated on the basis of the HMBC spectrum, which showed the correlation of H-3 (δ 6.71) and GlCUA H-1 (δ 4.66)/C-1 (δ 142.4) (Supplemental Fig. S17). The upfield shifted GlCUA H-1 (δ 4.66 versus 5.07, MI14), caused by the anisotropic effect of the bottom ring, also supported the location of this conjugate (Supplemental Table S2). MI5 (4R-hydroxylastourvilline 1-0-β-glucuronide) is a 4β-hydroxylated analog of MI8, as exemplified by the MS data and similar 1H NMR data except for the H-4 signals, a broad singlet (δ 4.86) in MI5 (Table 2). The NOESY spectrum showing the correlation of H-3/H-4, H-8/9-OMe, and H-11/10-OMe also supported this structure assignment.

**2-O-Glucuronides (MI4, -6, and -7).** MI4 (4R-hydroxylastourvilline 2-0-β-glucuronide) had the same molecular formula as MI5 (Table 4) and is the structure isomer of MI5 with exchanged substituents at C-1 and C-2 as verified by the 1H NMR data. They have similar 1H NMR data except for the downfield shifted H-3 (+0.29 ppm) and H-11 (+0.12 ppm) in MI4 relative to the corresponding signals in MI5 (Table 2). Likewise, MI7 (lastourvilline 2-0-β-glucuronide) had the same molecular formula as MI8 (Table 4) and is the structure isomer of MI8 with exchanged substituents at C-1 and C-2 as supported by the similar 1H NMR spectra with difference for the downfield shifted H-3 (+0.28 ppm), H-11 (+0.11 ppm), and GlCUA H-1 (+0.27 ppm) in MI7 relative to the corresponding signals in MI8 (Supplemental Table S2). The structure for MI7 was confirmed by the NOESY spectrum (Supple-
### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
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### Discussion

This work led to the characterization of 24 metabolites of dicentrine (1) from the urine of miniature pigs, including 9 phase I metabolites (MII-1–9) and 15 phase II conjugates (MII-1–15). Structural analysis indicates that these phase I metabolites were produced via the generally recognized pathways including C-4 benzylic hydroxylation (MII-1–3 and -5–7), subsequent O-methylation (MII-3), N-oxidation (MII-7), N-demethylation (MII-8), O-demethylation (MII-2, -5, -6, -8, and -9), and O-demethylation (MII-1, -3, and -4) (Fig. 1).

The amounts of the phase II metabolites were found to be much more than those of the phase I metabolites in the urine, as expected by the facile formation of the glucuronide conjugates to facilitate the
elimination. The major metabolite MII-15, as observed from the HPLC-UV chromatogram of fraction B-I (Fig. 4), was the 9-O-glucuronide of metabolite MII-8. It is noted that the phenolic group not only undergoes common glucuronidation but also glucosylation as observed in MII-2, similar to that reported for mycophenolic acid (Shipkova et al., 2001). The structures of the metabolites MII-11–13 also point to the oxidation at the C-3 position in the aryl ring as being one of the phase I transformations (Fig. 3), although the corresponding phase I metabolites were not detected in this study.

Few studies on the metabolism of aporphines have been reported. For example, four phase II metabolites of apomorphine, a drug used in the treatment of Parkinson’s disease, had been identified in vitro and in rat urine (Keski-Hynnilä et al., 2002) and also in human urine (van der Geest et al., 1998). They were characterized as apomorphine 9-O-sulfate, 9-O-glucuronide, 10-O-sulfate, and 10-O-glucuronide by HPLC-MS or HPLC-electrochemical detector. Our study revealed the phase II transformation of dicentrine by glucuronidation in miniature pig. No sulfate conjugates were detected in the present study. The in vitro metabolic study of thalicarpine, a dimeric aporphine and benzyltetrahydroisoquinoline alkaloid, indicated three thalicarpine and benzylic oxidation/reduction (Wu and McKown, 2002). The in vivo metabolic pathways of dicentrine revealed from our study are generally consistent with those observed in thalicarpine and apomor-
phenal except for O-sulfation, probably attributable to the species difference.

Based on the HPLC-DAD-MS analysis, all of the metabolites identified were from urine, but neither metabolites nor the parent compound were detected from the feces collected during the same period. The result showed that dicentrine mesylate was absorbed well through the gastrointestinal tract and eliminated predominantly through the kidney in the miniature pig.

This study characterized six pairs of structural isomers, including MI-2/MI-5, MI-3/MI-6, MIII-3/MII-14, MIII-4/MII-5, MIII-7/MII-8, and MII-9/MII-12. The structures of these isomeric metabolites were determined by the analysis of MS and informative 1D/2D NMR data. The HPLC-SPE-NMR technique, which requires only submilligram amounts of the desired sample mixture, obtained after appropriate sample pretreatment and focusing, but a baseline resolved HPLC condition, could provide well resolved 1H NMR and 1H-detected 2D NMR spectra for each compound, accelerating greatly for the identification of drug metabolites. Miniature pig, a much larger animal than the commonly used rat, could be administered more samples and therefore provide larger amounts of metabolites, allowing

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**Fig. 3.** Proposed phase II metabolic pathways of dicentrine.

**Fig. 4.** Reverse-phase HPLC chromatogram of fraction B-1, monitored at UV 280 nm (for other liquid chromatography conditions, see Materials and Methods). mAU, milli-absorbance units.
their separation in a semipreparative scale and facilitating their unambiguous structural elucidation by general spectroscopic analysis. These metabolites might be active or toxic. Thus, the preparation of these metabolites would be essential for further clarification of such issue(s) and for exploration of new drug candidates.

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References


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