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Pharmacokinetics and Urine Metabolite Identification of Dehydroevodiamine in the Rat

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ABSTRACT: This study investigates the oral bioavailability and characterizes urine metabolites of dehydroevodiamine (DeHE), one of the bioactive alkaloids isolated from the fruit of *Evodia rutaecarpa*. A freely moving rat model coupled with an automated blood sample system was used to evaluate the pharmacokinetics of DeHE. High-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectrometry were applied to determine DeHE and its metabolites. The averaged oral bioavailability of DeHE (100 and 500 mg/kg) in the freely moving rats was approximately 15.35%. Cumulative fecal and urinary excretions of unchanged DeHE were 6 and 0.5%, respectively, after a single oral dose (500 mg/kg) of DeHE. The protein binding of DeHE in rat plasma was 65.6 ± 6.5%. Six metabolites, including five DeHE−*O*-glucuronides and one DeHE−sulfate, were identified after oral administration. The structures of two glucuronide conjugates, DeHE−10-*O*-glucuronide (M3) and DeHE−11-*O*-glucuronide (M4), and one sulfate conjugate, DeHE−12-sulfate (M6), were assigned. The findings indicate that the oral bioavailability of DeHE was much higher than that of evodiamine, and hydroxylation and conjugative metabolism were essential for the urinary elimination of DeHE.

KEYWORDS: *dehydroevodiamine, oral bioavailability, metabolism, pharmacokinetics*

INTRODUCTION

Dehydroevodiamine (DeHE, Figure 1) is isolated from the unripe fruit of *Evodia rutaecarpa* (Juss.) Benth. (a Chinese medicinal herb named Wu-chu-yu), which has been used to alleviate abdominal pain, acid regurgitation, nausea, diarrhea, and dysmenorrheal.[1] A number of quinazolinocarboline alkaloids have been isolated from Wu-chu-yu, including DeHE, evodiamine, rutaecarpine, rutaevine, wuchuyine, and rhetsinine.[1−3] DeHE attenuates learning and memory impairments caused by chemicals or cerebral ischemia in rodents.[4−6] DeHE also attenuates calyculin A-induced τ-hyperphosphorylation at multiple Alzheimer's disease-related sites in rat brain slices[7] and improves spatial memory deficit in rats.[8]

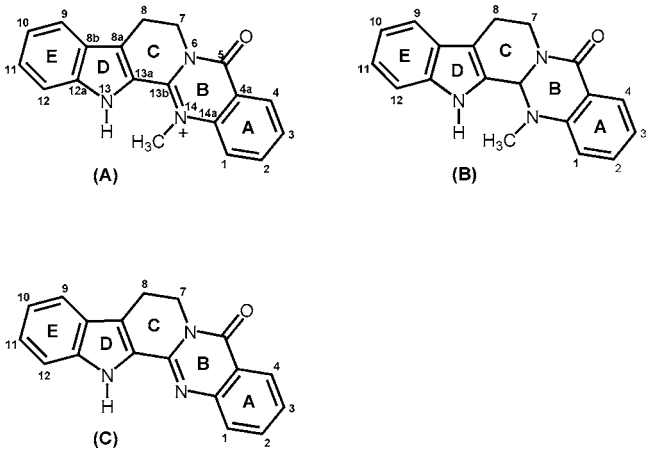

Figure 1. Chemical structures of (A) dehydroevodiamine (DeHE), (B) evodiamine, and (C) rutaecarpine.

Although DeHE has many pharmacological activities, the information of DeHE pharmacokinetics is still limited. A better understanding for the absorption, distribution, metabolism, and elimination of herbal products is necessary to design rational dosage regimens.[9] To reflect the absorption and the first pass effect with which an herbal ingredient is absorbed after oral administration, its bioavailability must be assessed. In addition, metabolic pathways and elimination routes of DeHE remain to be clarified. The determination of DeHE in plasma and bile has been reported,[10] but its pharmacokinetic parameters were not described. The plasma concentration profiles versus time were found to be compatible with a two-compartment model and a first-order kinetic when DeHE was examined via intravenous (iv) administration.[11] The pharmacokinetics of DeHE in the rat brain and plasma has been evaluated, showing that DeHE is transported from the systemic circulation to the brain across the blood−brain barrier by linear kinetics.[12] To date, no definite oral bioavailability of DeHE has been proposed, and limited data were reported to describe the metabolic pathways and elimination of DeHE. The major aims of the present study are to investigate oral bioavailability, to examine regional brain

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distribution, and to identify the main metabolites of DeHE in the rat urine.

MATERIALS AND METHODS

Chemicals and Reagents. DeHE (purity = 98% verified by HPLC) was purified and identified by our laboratory.[3] Liquid chromatographic grade solvents were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Purified water (Millipore, Bedford, MA) was used for all preparations. The stock solution of DeHE was prepared in acetonitrile and stored at −20 °C for method validation. Colchicine solution (1 μg/mL) prepared in methanol was used as the internal standard for DeHE quantification.

Animal Preparations. Male specific pathogen-free Sprague−Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei. All animal experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of National Yang-Ming University. To evaluate oral bioavailability, rats (280−320 g) were initially anesthetized with pentobarbital (50 mg/kg) and remained anesthetized throughout the operation. During surgery the rats' body temperature was maintained with a heating pad. Catheterization was performed as described earlier.[13] For the intravenous administration group (n = 6), surgical sites were shaved and cleaned with 70% ethanol solution, and two polyethylene catheters were implanted in the right jugular and right femoral veins, respectively. For the oral administration group (n = 6), only the right jugular vein was catheterized for blood sampling. The catheter of the right jugular vein was exteriorized, fixed in the dorsal neck region, and connected to the automated blood sampling system (DR-II, Eicom, Kyoto, Japan). The patency of the tubing was maintained by flushing with heparinized saline (15 IU/mL). Rats were allowed a minimum of 24 h to recover from surgery.

HPLC-UV Assay. The HPLC system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN), a manual injector (Rheodyne 7725, Cotati, CA) with a 20 μL sample loop, and an ultraviolet detector (S3702, Soma, Tokyo, Japan). An Inertsil ODS3-C18 column (250 × 4.6 mm;, 5 μm; Merck, Darmstadt, Germany) was used to separate DeHE in the plasma, urine, fecal, and brain samples. The mobile phase consists of 30% acetonitrile and 70% 10 mM aqueous NaH_2PO_4 (adjusted to pH 2.5 by the addition of pure orthophosphoric acid), and the flow rate was 1.0 mL/min. The detection wavelength for DeHE was set to 360 nm. Output data from the detector were processed by an EZChrom chromatographic data system (Scientific Software, San Roman, CA). Linearity, accuracy, and precision were evaluated to validate the HPLC method. All calibration curves were required to have a coefficient of determination (r^2) of at least 0.995. The intra- and interassay variabilities were determined by quantitating six replicates at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 50 μg/mL using the HPLC method described above on the same day and six consecutive days, respectively. The lowest concentration of the linear regression defined the limit of quantitation (LOQ). The accuracy (% bias) was calculated from the mean value of observed concentration (C_{obs}) and the nominal concentration (C_{nom}) using eq 1:

$$\text{accuracy (\%bias)} = [(C_{obs} - C_{nom})/C_{nom}] \times 100 \quad (1)$$

The relative standard deviation (RSD) was calculated from the observed concentrations using eq 2:

$$\text{precision (\%RSD)} = [\text{standard deviation(SD)}/C_{obs}] \times 100 \quad (2)$$

Oral Bioavailability Assessment. The automated blood sampling system DR-II (Eicom Kyoto, Japan) has previously been applied for blood sampling in freely moving rats.[14,15] DeHE was dissolved in polyethylene glycol 400 and ethanol (4:1, v/v) for drug administration. For the iv group, DeHE (10 mg/kg) was given via the femoral vein catheter, whereas DeHE (100 or 500 mg/kg) was given via gastric gavage for the oral groups. One hundred and fifty microliters of blood from the jugular vein catheter was withdrawn into a heparin-rinsed vial refrigerated in a fraction collector according to a programmed schedule, and the lost blood was replaced with an equal volume (150 μL) of heparinized saline after each sampling. In the group of intravenous administration, blood samples were collected at 1, 5, 10, 20, 30, 40, and 50 min and 1, 1.5, 2, 2.5, 3, and 3.5 h after dosing. At the lower oral dose of 100 mg/kg, blood samples were collected at 15, 30, and 45 min and 1, 1.5, 2, 3, 4, 5, 6, 8, 9, 10, and 12 h after administration. In the oral 500 mg/kg group, sampling time was extended to 72 h after administration. Each blood sample was centrifuged at 3000g for 10 min to acquire the plasma. The plasma sample (60 μL) was vortex-mixed with 180 μL of methanol containing colchicine (1 μg/mL) as the internal standard, and the supernatant separated by centrifugation at 8000g for 10 min was analyzed by HPLC-UV.

Brain Regional Distribution and Plasma Protein Binding. Rats (n = 6) under anesthesia received DeHE (10 mg/kg) via the femoral vein catheter. Blood was collected by cardiac puncture at 1 and 5 min after administration, and samples were centrifuged at 6000g for 10 min to obtain supernatant plasma. A 60 μL sample of plasma was used to determine the total form concentration (C_t) of the drug. A 300 μL plasma sample was centrifuged by an Ultrafree-MC ultrafiltration unit (Millipore, Bedford, MA) with a 30000 molecular mass cutoff at 2000g under 4 °C for 30 min to obtain the filtrate,[16] which was processed using the same procedures of plasma samples by HPLC-UV to acquire the unbound drug concentration (C_u). The protein-bound ratio of the drug was calculated by eq 3:[17]

$$\text{protein binding (\%)} = [(C_t - C_u)/C_t] \times 100\% \quad (3)$$

Immediately after blood collection, the rats were sacrificed by decapitation, and then the cortex, hippocampus, cerebellum, brainstem, striatum, and remainder of the brain were divided and weighed. Each type of brain tissue was homogenized with cold methanol (50%, v/v) at the ratio of 1:5 (g/mL). The brain homogenate was then centrifuged at 3000g for 10 min, and the cold methanol extract (60 μL) was vortex-mixed with 180 μL of methanol containing colchicine (1 μg/mL). The brain supernatant was separated by centrifugation at 8000g for 10 min before HPLC analysis.

Fecal and Urinary Excretion of DeHE. The urine and fecal samples were separately collected at 12 h intervals for 5 consecutive days by a metabolic cage (Mini Mitter, Bend, OR) after oral administration of DeHE (500 mg/kg, n = 6). During this experiment, the rats were also allowed free access to food and water in the animal facilities. Urine samples were first centrifuged at 3000g for 10 min, and the primary supernatant (60 μL) was processed according to the same procedures as for plasma samples. The urine supernatant was obtained by centrifugation at 8000g for 10 min. The fecal sample was extracted with methanol (1 g: 20 mL, w/v) by ultrasonication for 20 min, and the methanol extract was filtered with filter paper (Whatman 5 filter paper, Maidstone, U.K.). The fecal meth nol extract (60 μL) was vortex-mixed with 180 μL of methanol containing colchicine (1 μg/mL) stored in −20 °C until analysis. The supernatant obtained by centrifugation of urine and fecal samples at 8000g for 10 min was analyzed by HPLC-UV. The recoveries for feces ranged from 96.6 to 102.3%, and the recoveries for urine ranged from 92.8 to 104.2%.

Isolation of the Urinary Metabolites. The collected urine (about 200 mL) from rats receiving DeHE (500 mg/kg) was subjected to a preparative column (MCI CHP 20P, 3 × 60 cm, Mitsubishi Chemical, Tokyo, Japan), washed with H_2O (700 mL), and then the retained sample was eluted with methanol (1 L). The concentrated methanol fraction (100 mL) was chromatographed on a column (Cosmosil 140 C_{18}-OPN, 60 × 5.5 cm, i.d., Nacalai Tesque, Kyoto, Japan), washed by H_2O (1 L), and finally eluted with 30% CH_3CN/H_2O (1 L). The 30% CH_3CN/H_2O fraction was evaporated to dry, dissolved in methanol, and subjected to HPLC separation. The HPLC was performed with a Cosmosil 5C18-AR-II column (250 × 4.6 mm, i.d., 5 μm, Nacalai Tesque). Acetonitrile and 1% acetic acid water solution (v/v) were used as mobile phases A and B, respectively. The gradient elution initiated at 10% mobile phase A, linearly changed to 30% mobile phase A within 30 min, and remained at 30% for a further 5 min with a flow rate of 1.2 mL/min. Chromatograms were

monitored and recorded at 360 nm. The intervals for collecting metabolites 1−6 were 2.5−4.0, 6.0−7.0, 7.5−8.0, 12.5−13.5, 14.5−15.5, and 24−26 min, respectively.

HPLC-MS/MS and NMR Conditions. Identification was carried out on a Waters 2690 HPLC system (Bedford, MA) with a 996 photodiode array detector, scanning from 200 to 600 nm. Separation was carried out using a column (Cosmosil 5C18-AR-II, 4.6 × 250 mm, 5 μm) eluted with a 30 min gradient of 10−30% acetonitrile in 1% acetic acid and held at 30% for a further 5 min at a flow rate of 1.0 mL/min. After passing through the flow cell of the diode array detector, the column elute was split, and 0.3 mL/min was directed to a mass spectrometer equipped with an electrospray interface (Quattro Ultima, Micromass, Manchester, U.K.). Analysis was carried out with full scan using positive ion mode and data-dependent MS/MS scanning from m/z 250 to 900. The instrument settings were as follows: capillary voltage, 3.0 kV; cone voltage, 50 V; source temperature, 80 °C; desolvation temperature, 370 °C. HPLC-MS/MS data were processed by the MassLynx version 4.0 NT Quattro data acquisition software (Micromass). High-resolution fast atom bombardment mass spectra (HR-FAB-MS) were recorded with a Finnigan/Thermo Quest MAT95XL spectrometer, with glycerol as a liquid matrix. NMR spectra were measured with a Varian Inova-500 spectrometer (Varian, Walnut Creek, CA). Samples were dissolved in deuterated solvents (methanol-d_4 or DMSO-d_6, CIL, MA). Chemical shifts are expressed in parts per million relative to deuterated solvent as an internal standard for NMR analysis.

Pharmacokinetic and Statistical Analysis. The concentrations of DeHE in plasma, urine, feces, and brain tissue were calculated from the corresponding calibration curves. The maximum plasma concentration (C_{max}), the time to reach C_{max} (t_{max}), the area under the plasma concentration versus the time curve (AUC), and clearance (Cl) were calculated using the noncompartmental model of the pharmacokinetic program WinNonlin Standard Edition version 1.1 (Scientific Consulting, Apex, NC). The oral bioavailability was calculated by eq 4:

$$\text{oral bioavailability (\%)} = (\text{AUC}_{po}/\text{dose}_{po})/(\text{AUC}_{iv}/\text{dose}_{iv}) \quad (4)$$

All data are expressed as the mean ± SD. Statistical analysis was assessed by Student's t test with $p < 0.05$ being considered significant.

RESULTS

Validation of HPLC-UV Assay. The chromatograms shown in Figure 2 indicated the good selectivity of the HPLC-UV method by observing blank samples (Figure 2A), and no interference or other metabolites coeluted with DeHE. The calibration curve for the determination of DeHE in plasma and feces was linear over the range 0.05−50 μg/mL. The calibration curve for urine samples was linear over the range 0.5−50 μg/mL. Peak area ratios of DeHE versus the internal standard (colchicine) were measured and plotted against the concentration of DeHE for the calibration curve. Each sample was prepared in six replicates, and seven different concentrations were used to determine linearity. The limits of detection (at the signal-to-noise ratio of 3) of DeHE in rat plasma, feces, and urine were 0.01, 0.01, and 0.1 μg/mL, respectively. The limits of quantification of DeHE in rat plasma, feces, and urine were 0.05, 0.05, and 0.5 μg/mL, respectively. The precision defined by the relative standard deviation (RSD) was within 14.6%. Analytical accuracy expressed as the bias was within −14.0 and 12.0%. Thus, the intraday and interday precision and accuracy were found to be acceptable for the DeHE analysis, in support of further pharmacokinetic studies. The data demonstrate excellent reproducibility. Mean drug recoveries were calculated by comparing the peak area ratios of extracted plasma, fecal, and urine samples with those obtained from unextracted calibrators with the same amount of DeHE. The extraction recoveries of DeHE in various biological samples were >91.4%.

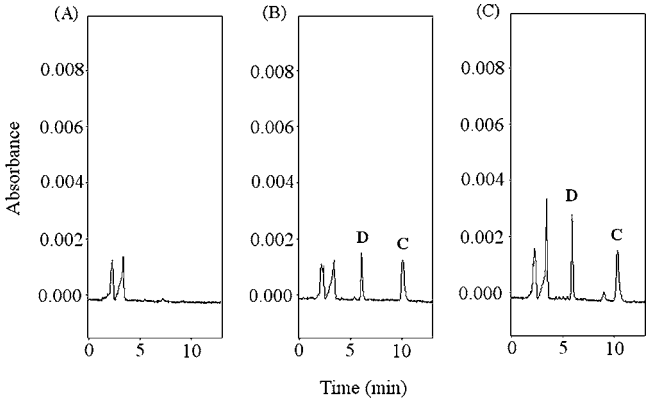

Figure 2. High-performance liquid chromatograms of (A) blank rat plasma, (B) blank rat plasma spiked with standard (DeHE, 1 μg/mL), and (C) rat plasma sample containing DeHE (1.5 μg/mL) collected at 9.5 h after oral administration (500 mg/kg). Peaks: D, dehydroevodiamine (DeHE, retention time = 5.8 min); C, colchicin (internal standard, retention time = 10.8 min).

Oral Bioavailability. The plasma DeHE concentration versus time curves are shown in Figure 3, and their corresponding pharmacokinetic parameters are summarized in Table 1. The AUC_{0-t} and C_{max} were 107 ± 38 min μg/mL and 5.4 ± 0.8 μg/mL, respectively. After oral administration at doses of 100 and 500 mg/kg, the AUCs were 177 ± 50 and 757 ± 147 min μg/mL, respectively. No significant difference of the AUCs normalized by the doses indicates a linear pharmacokinetic behavior during the dosage range. The oral bioavailabilities for 100 and 500 mg/kg administrations of DeHE in freely moving rats were 16.7 and 14.3%, respectively.

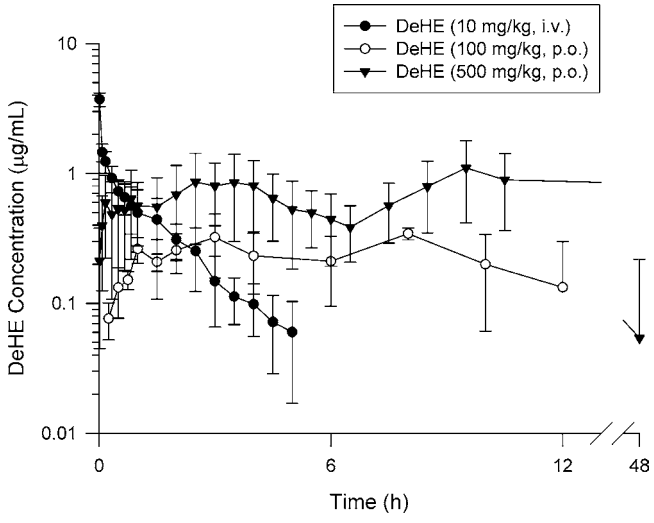

Figure 3. Average blood concentration−time curves of DeHE after intravenous administration of DeHE (10 mg/kg) (●), oral administration of DeHE (100 mg/kg) (○), and oral administration of DeHE (500 mg/kg) (▼) to rats. Data are expressed as the mean value ± SD (n = 6 for each group).

Brain Regional Distribution and Plasma Protein Binding. The plasma concentration of DeHE was 4.82 ± 1.55 μg/mL,

Table 1. Pharmacokinetic Data of DeHE after DeHE Administration in Rats[a]

parameter	10 mg/kg, iv	100 mg/kg, po	500 mg/kg, po
AUC (min μg/mL)	107 ± 38	177 ± 50	757 ± 147
C_0 or C_{max} (μg/mL)	5.4 ± 0.8	0.4 ± 0.1	1.7 ± 0.3
Cl (mL/kg/min)	110 ± 16	547 ± 130	683 ± 142
oral bioavailability (%)		16.5	14.2

[a]Data are expressed as the mean ± SD. iv, intravenous; po, per oral; AUC, area under the plasma concentration versus the time curve, C_0, plasma drug concentration at time = 0; C_{max}, maximum plasma concentration; Cl, clearance.

and the concentrations of DeHE were 1.11 ± 0.4, 0.93 ± 0.24, 0.64 ± 0.28, 1.13 ± 0.33, 1.04 ± 0.3, and 1.18 ± 0.18 μg/g for cortex, hippocampus, striatum, cerebellum, brain stem, and the remainder of the brain, respectively, after 15 min of DeHE administration (10 mg/kg, iv). This result indicated that DeHE crossed the blood−brain barrier, and the concentrations of DeHE in rat plasma was 3−4-fold higher than those in the brain tissue. The average protein binding of DeHE was 65.6 ± 6.5%.

Fecal and Urinary Excretion. The average percentages of fecal recoveries at 0−24 and at 0−120 h were 2 and 6%, respectively. The unchanged DeHE recovered from urine is only 0.4%.

Identification of Urinary Metabolites. Six metabolites, M1−M6, were isolated by preparative HPLC (Figure 4). The UV absorption spectra (λ_{max} 360 nm) indicated that metabolites possessed the same quinazolinocarboline skeleton as DeHE. M1−M5 had similar mass spectra with molecular ion $[M]^+$ at m/z 494, and the representative mass spectra are shown in Figures 5−9. Mass spectra of M1−M5 also exhibited the same important fragment ions at m/z 318 $[M + H - C_6H_9O_6]^+$ and 302 $[M + H - C_6H_9O_7]^+$, deriving from a loss of glucuronyl group and glucuronic acid group from the molecular structure, respectively. On the basis of the UV and MS data, compounds M1−M5 are proposed to be DeHE−*O*-glucuronide.

The molecular formula of M3 was further established as $C_{25}H_{24}O_8N_3$ by high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) (found m/z 494.1568, calcd 494.1564), coinciding with the molecular formula of DeHE−*O*-glucuronide. The ^{1}H NMR spectrum (Table 2) of M3 showed the presence of seven aromatic proton at δ 8.06 (1H, br d, H-1), 8.12 (1H, br t, H-2), 7.77 (1H, t, J = 7.0 Hz, H-3), 8.43 (1H, d, J = 7.0 Hz, H-4), 7.52 (1H, s, H-9), 7.36 (1H, d, J = 8.0 Hz, H-11), and 7.55 (1H, d, J = 8.0 Hz, H-12). The aromatic signals of M3 were similar to those of DeHE (Table 2) except for protons in the E-ring. A ^{1}H−^{1}H COSY experiment showed clear coupling connections around the A- and E-rings, and assignment of protons was achieved. A singlet proton H-9 (δ 7.52) and a pair of ortho-coupling protons H-11 (δ 7.36) and H-12 (δ 7.55) were observed, suggesting that a substituent is attached to the C-10 or C-11 position. A survey of the literature data[18] found that H-9 and H-12 of indoles have lower field shifts than H-10 and H-11, and H-9 is located at the lowest field shift in ring E protons. Therefore, M3 was deduced as being C-10 substituted. Counting the effect of ortho-substituted and meta-substituted on the chemical shift of DeHE also confirmed above deduction. Integrating the mass, NMR data. and literature data, compound M3 was identified as DeHE−10-*O*-glucuronide.

The molecular formula of M4 was established as $C_{25}H_{24}O_8N_3$ by HR-FAB-MS (found m/z 494.1567, calcd 494.1564). The ^{1}H NMR spectrum of M4 showed the presence of seven aromatic proton at δ 8.04 (1H, d, J = 9.0 Hz, H-1), 8.10 (1H, dd, J = 9.0, 7.0 Hz, H-2), 7.77 (1H, t, J = 7.0 Hz, H-3), 8.42 (1H, d, J = 7.0 Hz, H-4), 7.73 (1H, d, J = 9.5 Hz, H-9), 7.08 (1H, d, J = 9.5 Hz, H-10), and 7.33 (br s, H-12). The aromatic signals of M4 resembled that of DeHE, but differed in the E-ring (Table 2). The ^{1}H−^{1}H COSY study suggested that a substituent is attached to the C-10 or C-11 position. High-field shifts of about 0.24−0.35 ppm were observed in H-10 and H-12 compared with those of DeHE, caused by ortho-substituted effect on the chemical shift, confirming C-11 substitution. On the basis of the mass (Figure 8) and NMR data, compound M4 was identified as DeHE−11-*O*-glucuronide.

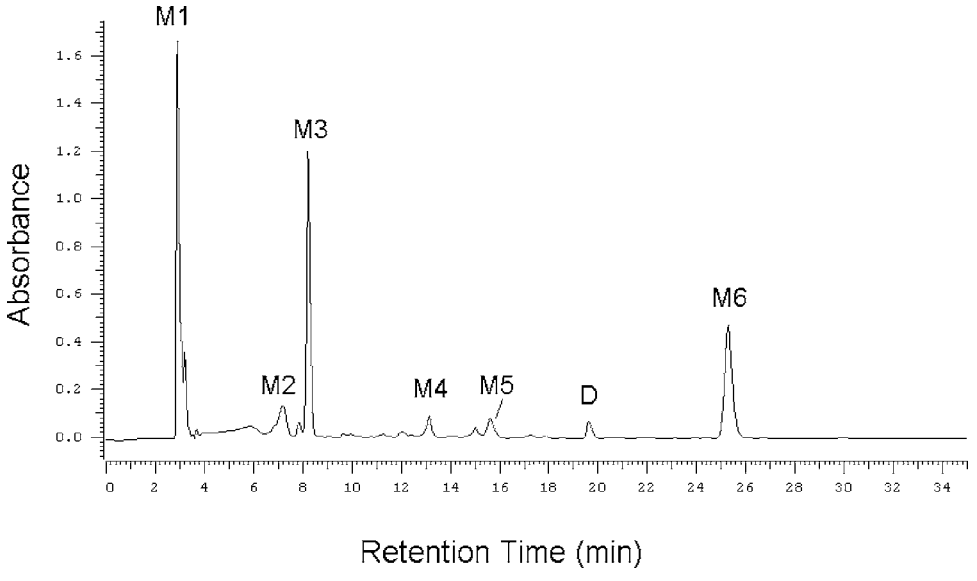

Figure 4. Representative HPLC chromatogram of the fractionated rat urine sample spiked with DeHE. Peaks: M1−M6 represent six metabolites, respectively; D, DeHE.

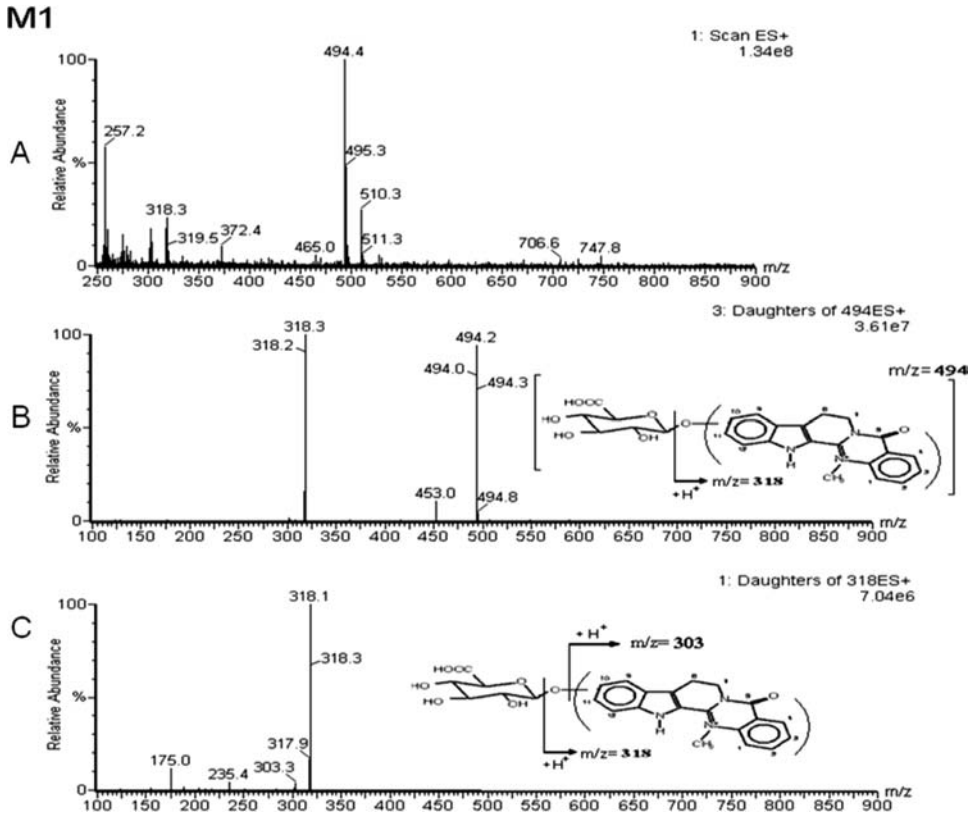

Figure 5. HPLC-MS/MS spectra and the proposed fragmentation scheme of M1: (A) MS spectrum; (B) MS^2 spectrum of m/z 494; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

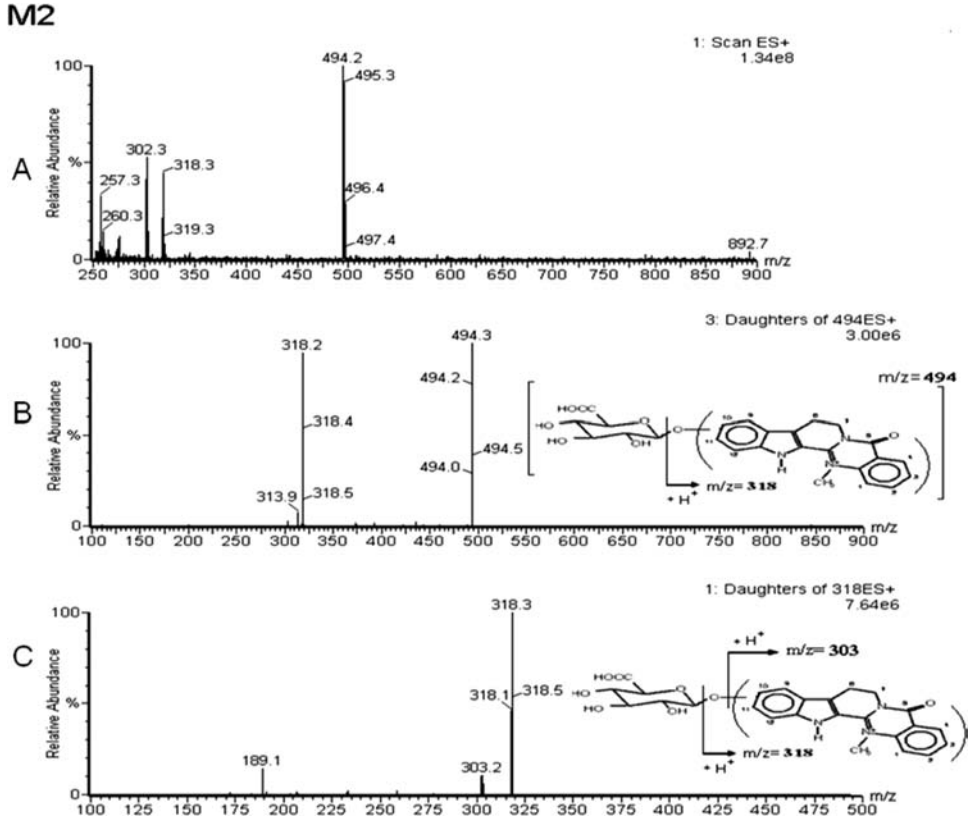

Figure 6. HPLC-MS/MS spectra and the proposed fragmentation scheme of M2: (A) MS spectrum; (B) MS^2 spectrum of m/z 494; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

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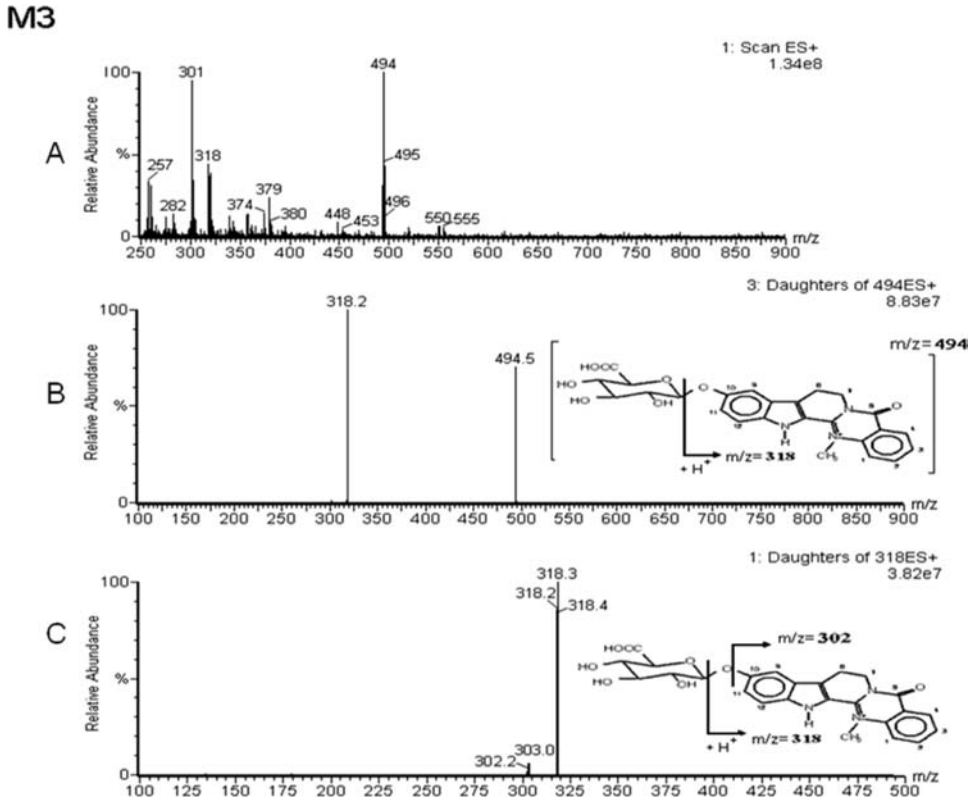

Figure 7. HPLC-MS/MS spectra and the proposed fragmentation scheme of M3: (A) MS spectrum; (B) MS^2 spectrum of m/z 494; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

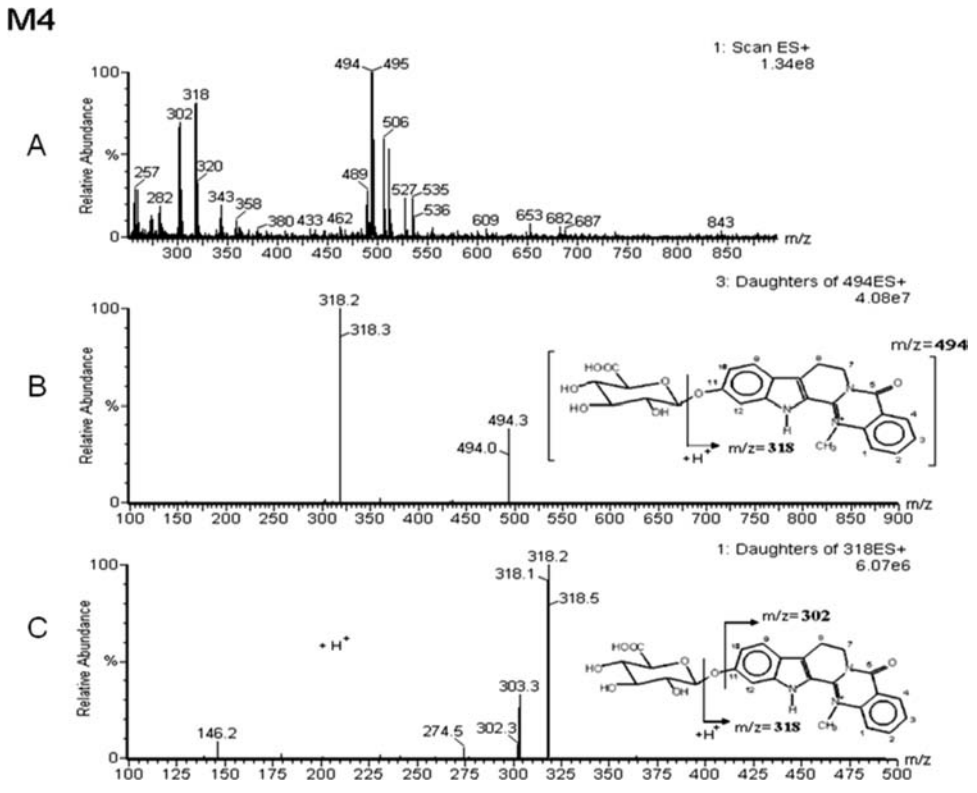

Figure 8. HPLC-MS/MS spectra and the proposed fragmentation scheme of M4: (A) MS spectrum; (B) MS^2 spectrum of m/z 494; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

In the HPLC-MS/MS analysis (Figures 5, 6, and 9), a fragment at m/z 318 $[M + H - C_6H_9O_6]^+$ deriving from ion m/z 494 and a fragment at m/z 303 $[318 + H - 16]^+$ deriving from ion m/z 318 were all observed in M1, M2, and M5,

dx.doi.org/10.1021/jf204365m | J. Agric. Food Chem. 2012, 60, 1595−1604

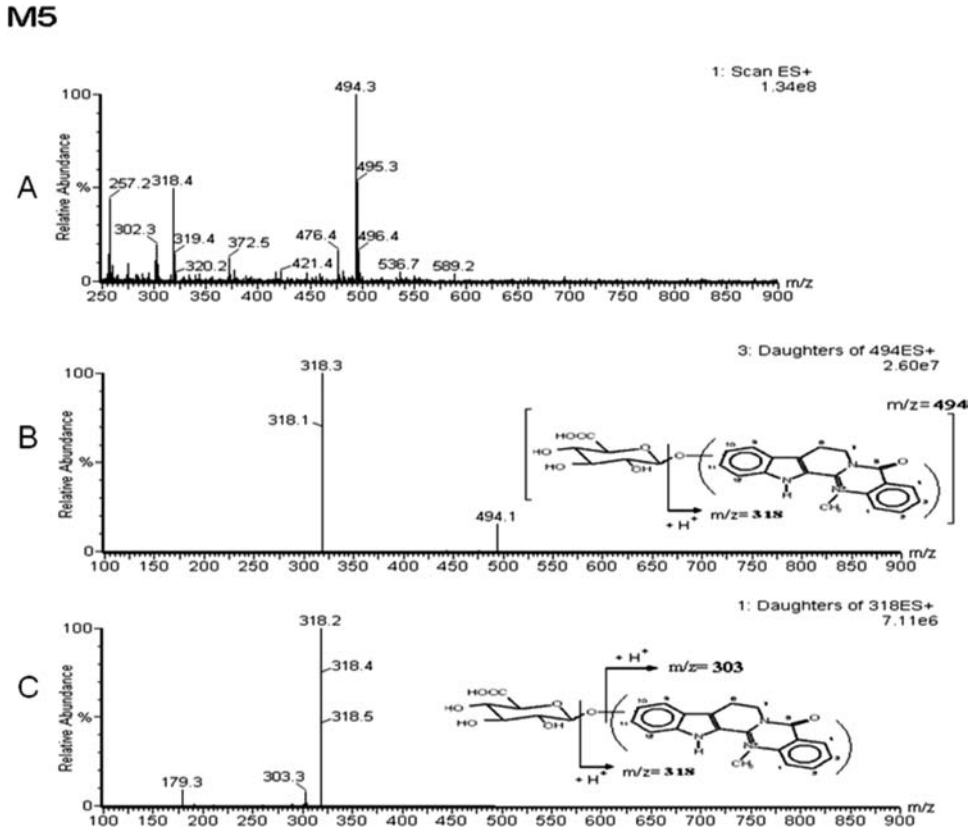

Figure 9. HPLC-MS/MS spectra and the proposed fragmentation scheme of M5: (A) MS spectrum; (B) MS^2 spectrum of m/z 494; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

Table 2. ^{1}H NMR Spectral Data of DeHE and DeHE Metabolites M3, M4, and M6

position	DeHE[a]	M3[a]	M4[a]	M6[b]
H-1	8.08 (d, J = 8.0)[c]	8.06 (br d)	8.04 (d, J = 9.0)	8.14 (d, J = 7.5)
H-2	8.13 (dd, J = 8.0, 7.5)	8.12 (br t)	8.10 (dd, J = 9.0,7.0)	8.12 (t, J = 7.5)
H-3	7.80 (t, J = 7.5)	7.77 (t, J = 7.0)	7.77 (t, J = 7.0)	7.78 (t, J = 7.5)
H-4	8.45 (d, J = 7.5)	8.43 (d, J = 7.0)	8.42 (d, J = 7.0)	8.33 (d, J = 7.5)
H-9	7.89 (d, J = 8.0)	7.52 (s)	7.73 (d, J = 9.5)	7.55 (d, J = 8.0)
H-10	7.32 (dd, J = 8.0, 7.5)		7.08 (d, J = 9.5)	7.15 (d, J = 8.0)
H-11	7.57 (t, J = 8.0)	7.36 (d, J = 8.0)		7.39 (d, J = 8.0)
H-12	7.68 (d, J = 8.0)	7.55 (d, J = 8.0)	7.33 (br s)	

[a]Measured in methanol-d_4. [b]Measured in DMSO-d_6. [c]Splitting pattern (s, singlet; d, doublet; dd, double doublet; t, triplet; br, broad); coupling constants in hertz are in parentheses.

indicating a loss of glucuronyl group and a greater loss of oxygen from the molecular structure. On the basis of the UV spectra and MS/MS data, the structures of M1, M2, and M5 were deduced as DeHE−*O*-glucuronides. Their structures just differ from the substituted position of glucuronic acid on the molecule. Due to insufficient amounts, the glucuronidated positions of M1, M2, and M5 were not assigned.

The metabolite M6 had a molecular ion at m/z 398 in ESI-MS (Figure 10), in harmony with a molecular formula of $C_{19}H_{16}O_5N_3S$, which was further confirmed by HR-FAB-MS (found m/z 398.0815, calcd 398.0811). The fragment ions at m/z 318 $[M + H - HSO_3]^+$ and 302 $[M + H - HSO_4]^+$ derived from a loss of sulfonyl group and a sulfuric acid group from the molecule, respectively, suggesting the presence of a sulfuric acid group in M6. The ^{13}C NMR (DMSO-d_6) spectrum of M6 displayed 19 signals, which were sorted by DEPT experiments to divide into a primary carbon at δ 41.2 (N−CH_3); two secondary carbons at δ 18.8 (C-8) and 42.0 (C-7); seven secondary carbons at δ 116.5 (C-9), 118.6 (C-1), 118.9 (C-11), 122.1 (C-10), 127.7 (C-4), 128.6 (C-3), and 136.7 (C-2); and nine quaternary carbons at δ 118.6 (C-4a), 120.4 (C-13a), 125.6 (C-8b), 131.0 (C-8a), 134.2 (C-12a), 139.8 (C-14a), 140.3 (C-12), 149.8 (C-13b), and 158.2 (C-5). The ^{1}H NMR spectrum showed the presence of seven aromatic protons at δ 8.14 (1H, d, J = 7.5 Hz, H-1), 8.12 (1H, t, J = 7.5 Hz, H-2), 7.78 (1H, t, J = 7.5 Hz, H-3), 8.33 (1H, d, J = 7.5 Hz, H-4), 7.55 (1H, d, J = 8.0 Hz, H-9), 7.15 (1H, t, J = 8.0 Hz, H-10), and 7.39 (1H, d, J = 8.0 Hz, H-11). The difference of the ^{1}H NMR spectra between M6 and DeHE was the arrangement of protons in the E-ring (Table 2). In the ^{1}H−^{1}H COSY experiment, cross peaks of H-9−H-10 and H-10−H-11 were observed, implying that a substituent was attached to the C-12 position. Key HMBC corrections were observed between H-9 (δ 7.55)/H-7 (δ 4.80, t, J = 7.0 Hz)/H-8 (δ 3.32, overlapped with solvent) and C-8a (δ 131.0), confirming the unsubstitution of C-9. Integrated mass and NMR data identified compound M6 as DeHE−12-sulfate.

DISCUSSION

The herbal extract of Wu-chu-yu and its active ingredient have been used to ameliorate gastrointestinal diseases and central

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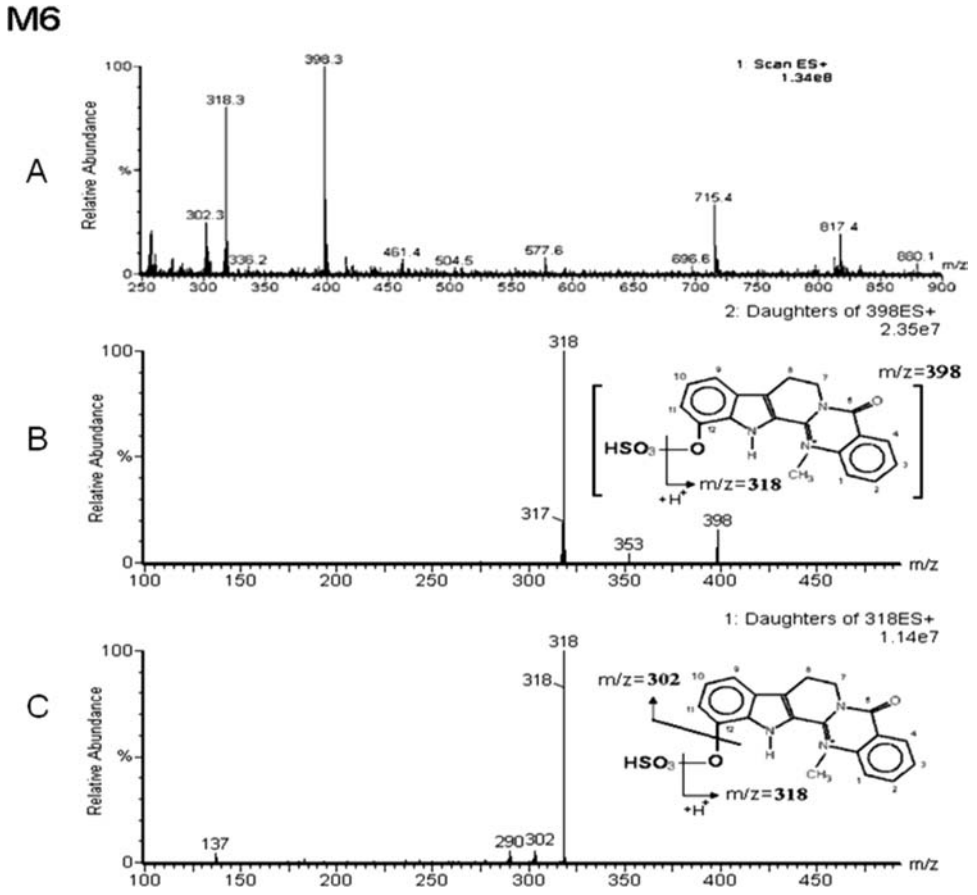

Figure 10. HPLC-MS/MS spectra and the proposed fragmentation scheme of M6: (A) MS spectrum; (B) MS^2 spectrum of m/z 398; (C) MS^2 spectrum of m/z 318. The MS^2 data were obtained by collision-induced dissociation.

nervous disorders, respectively.[1,4−8] We first demonstrate a freely moving rat model to investigate the oral bioavailability and brain distribution of DeHE to support the above pharmacodynamic effects. The use of anesthetic agents and immobilization stress may affect gastrointestinal functions and may influence the absorption and metabolism of drugs in rodents.[19,20] An automated blood sampling system for pharmacokinetic studies has been proposed to eliminate the influence of anesthesia and minimize the immobilization stress of the experimental animals.[21,22] Therefore, we select the automated blood sampling system to evaluate the absorption of DeHE in freely moving rats. The oral bioavailability of DeHE was approximately 15.35%, much higher than that of evodiamine (0.1%) in the previous paper.[23] However, the in vitro data demonstrate that evodiamine has been well absorbed by the permeability coefficients in a human colon adenocarcinoma cell line model.[24] Our animal studies have found diverse results for the oral bioavailabilities of evodiamine and DeHE.

Numerous time-dependent and variable factors in the gastrointestinal tract may affect the drug absorption from the intestinal tract.[25,26] Irregular absorption of DeHE in the gastrointestinal tract may result in the appearance of double peaks in plasma concentration−time profile and affect the time to reach maximal plasma concentration (t_{max}) after oral administration of DeHE. Possible reasons for the double-peak phenomenon have been proposed, such as the enterohepatic circulation and the irregular gastric emptying pattern.[27] Enterohepatic recycling could be precluded from being the mechanism of the double-peak phenomenon in our study, because double peaks appear after the oral doses but not after the intravenous dose.[28] The quaternary ammonium structure of DeHE could be a suitable subtract of organic cation transporters expressed in the intestine of the rat,[29] so DeHE might be actively secreted in the intestine to cause irregular absorption patterns. In the case of evodiamine, the inhibition of gastric emptying and gastrointestinal transit[30] might reduce its own absorption in rats.

In this study, we found that the brain concentration (approximately 1.0 μg/mL) was one-fifth of the plasma concentration (4.8 μg/mL) after an intravenous administration (10 mg/kg) of DeHE. We applied the automated blood sampling system to assess the oral bioavailability and pharmacokinetics of DeHE. The brain concentration of DeHE could be expected to be lower in rats receiving oral administration of DeHE than in rats receiving intravenous administration of DeHE, because the oral BA was only 15.35%. The metabolites of DeHE might play important roles on the neuroprotective effects,[4−6] and we further performed the identification of metabolites of DeHE. A previous paper demonstrates the metabolism of rutaecarpine, a quinazolinocarboline alkaloid, is mainly managed by cytochrome P450 1A2, and 10-, 11-, and 12-positions were considered the main sites for metabolic reactions.[31−34] The study of microbial metabolism of evodiamine also revealed that 10-hydroxyevodiamine and 11-hydroxyevodiamine were the main metabolites, which showed strong cytotoxic activity against several human cancer cell lines.[35] We found that hepatic first-pass metabolism may play an important role for DeHE elimination as supported

partly by the high clearance of DeHE.[36] In addition, the total amount excreted to the urine and the bile was <15% of the dose after an intravenous administration of DeHE,[12] suggesting that metabolism was the major route of DeHE elimination.

DeHE first underwent phase-I hydroxylation followed by conjugations. Mass spectra of metabolites M1−M5 showed the same molecular ion at m/z 494. Characteristic peaks at m/z 318 and 302 were also found in these spectra, which were likely caused by the in-source dissociation of glucuronides in electrospray ionization.[37] The product ions of m/z 494 generated by collision-induced dissociation (CID) produced a major peak at m/z 318, which was consistent with the loss of 176 Da, suggesting the molecular weight of glucuronic acid.[38,39] Similarly, the product ions of m/z 318 generated by CID produced a major peak at m/z 302, which was in agreement with the loss of 16 Da, suggesting the molecular weight of a hydroxyl group. For the identification of M6, the precursor ion at m/z 398 is 80 Da higher than the ion at m/z 318 formed from the hydroxylated DeHE, which demonstrated the loss of 80 Da (SO^{3-}), indicating the formation of sulfate conjugation.[40] According to the above data, metabolites M1−M5 are proposed to be DeHE−*O*-glucuronide and M6 is DeHE−sulfate. By the ^{1}H NMR and ^{1}H−^{1}H COSY experiments, the structures of M3, M4, and M6 are determined as DeHE−10-*O*-glucuronide, DeHE−11-*O*-glucuronide, and DeHE−12-sulfate, respectively. We have traced that the potential metabolic mechanism of DeHE may go through phase II glucuronidation and sulfation. However, the pharmacological activities of glucuronide metabolites of DeHE need to be clarified. Hence, the forthcoming study should focus on the detailed tissue distribution, metabolism, and pharmacodynamics of DeHE.

In conclusion, the oral bioavailability of DeHE in the freely moving rats was about 15.35% and was higher than that of evodiamine in the freely moving rat model. Two kinds of conjugation metabolites were found: *O*-glucuronide conjugate and sulfate conjugate. The metabolic pathways of DeHE included phase I reaction and phase II conjugation. The structures of two glucuronide metabolites, DeHE−10-*O*-glucuronide (M3) and DeHE−11-*O*-glucuronide (M4), as well as one sulfate conjugate, DeHE−12-sulfate (M6), were determined. Phase II conjugative metabolism was very important for the urinary elimination of DeHE, and the 10-, 11-, and 12-positions are the predominant positions for glucuronidation and sulfation.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DeHE, dehydroevodiamine; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; M3, DeHE-10-*O*-glucuronide; M4, DeHE-11-*O*-glucuronide; M6, DeHE-12-sulfate; LOQ, limit of quantitation; C_t, total form concentration; C_u, unbound drug concentration; C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; AUC, area under the plasma concentration versus the time curve; Cl, clearance; RSD, relative standard deviation; HR-FAB-MS, high-resolution fast atom bombardment mass spectrometry; CID, collision-induced dissociation.

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