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Relative Determination of Dehydroevodiamine in Rat Plasma by LC–MS and Study on its Pharmacokinetics

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The lack of purified standards is a bottleneck on assaying herbs *in vitro* and *in vivo*. This present work proposed a strategy of relative quantification that used a herb extract as a relative standard. A rapid and selective liquid chromatography tandem mass spectrometry method was similarly developed and validated for the relative determination of dehydroevodiamine in rat plasma according to the absolute quantification. Protein precipitation was used for the pretreatment of plasma samples. Chromatographic separation was achieved on a Diamonsil C18 column with an isocratic mobile phase of a 70:30 (v/v) acetonitrile–0.3% formic acid mixture at a flow rate of 0.45 mL/min. The assay was validated in the range 100.0 ∼ 50,000.0 ngH/mL ($r^2 = 0.9804$), the lowest level of this range being the lower limit of quantification based on 50 μL of plasma. The precision and accuracy were within recommended limits of nominal values. The method was applied to evaluate the comparative pharmacokinetics of dehydroevodiamine in rats following oral administration of *Evodia rutaecarpa* and *Rhizoma coptidis–Evodia rutaecarpa* couple. This approach was found to be capable of providing complete pharmacokinetic parameters as well as the typical pharmacokinetic assay calibrated by authentic standards, except for the absolute plasma concentrations.

Introduction

In the clinical practice of traditional Chinese medicine (TCM), most herbal medicines are prescribed in combination to obtain the synergistic effects or antagonistic action. *Rhizoma coptidis–Evodia rutaecarpa* couple (*R–E* couple) is one of the most widely used combination as the core in many composite formulae (1). Zuojinwan, which consists of *R–E* powder (6:1, g/g), is very effective for the treatment of gastrointestinal disorders. *Rhizoma coptidis* is generally regarded as the remedium cardinale in the combination and has been extensively studied (2–4), while very little attention has been devoted to *Evodia rutaecarpa*, although *Evodia rutaecarpa* also plays an indispensable role for the therapeutic effect in the combination. According to *Essentials of Matea Medica*, that the warm nature of *Evodia rutaecarpa* could attenuate the bitter cold nature of *Rhizoma coptidis* to protect the stomach from the repulsion between illness and medicines.

Evodiamine, rutecarpine and dehydroevodiamine are usually recognized as the most important pharmacologically active constituents in *Evodia rutaecarpa* (5). Methods have been described for evodiamine (6) and rutecarpine (7) determination in biological samples. Although dehydroevodiamine has many broad therapeutic effects (8–11), as far as we know, no method has been published for the determination or pharmacokinetic profiles of dehydroevodiamine in biological fluids.

The pharmacokinetic research of TCM has been seriously blocked by the lack of purified standards. It is impracticable to separate the constituents one by one to obtain standard substances for assays. The present paper demonstrates a relative reference approach in which a herb extract, instead of a purified standard, acted as the reference substance. On this basis, a rapid liquid chromatography–tandem mass spectrometry (LC–MS-MS) method for the determination of dehydroevodiamine in rat plasma was developed and applied to its pharmacokinetic after oral administration of *Evodia rutaecarpa* and *R–E* couple powders to rats independent of authentic standards.

Experimental

Materials

Methanol, formic acid and acetonitrile were of chromatographic grade from the Yuwang Chemical Factory (Shandong, China). Deionized water was purified by use of an Alpha-Q water-purification system (Millipore, Bedford, MA) for the preparation of samples and buffer solution. All other reagents were of analytical grade. *Rhizoma coptidis* and *Evodia rutaecarpa* were purchased from the Sifang Pharmacy (Shenyang, China).

Instrumentation and analytical conditions

Qualitative analysis was operated on a Thermo-Electron LCQ linear ion-trap mass spectrometer (Thermo-Electron, San Jose, CA) fitted with an electrospray ionization source over the mass range from m/z 50 to 2,000 in the positive ionization mode. Xcalibur 1.2 data analysis system was used. The spray voltage was set to 4.2 kV. The capillary voltage was fixed at 13 V. The heated capillary temperature was fixed at 210°C. Nitrogen used as the sheath and the auxiliary gas was set to 70 and 20 arbitrary units, respectively. The isolation width for MSn was 1.0 Da. The HPLC system consisted of an Agilent 1100 series equipped with a Agilent 1100 series photodiode-array detector (PDA) and autosampler Data Analysis (Agilent, Palo Alto, CA). Chromatographic separation was carried out on a Diamonsil C18 (150 × 4.6 mm i.d., 5 μm, Dikma) with an EasyGuard C18 Security Guard column (8 × 4.0 mm i.d., Dikma). The mobile phases consisted of 5 mM ammonium formate (0.025% formic acid) (A) and acetonitrile (B) using a gradient elution of 25% B at 0 min, 30% B at 5 min, 40% B at 15 min and 55% B at 20 min (v/v), at a flow rate of 0.5 mL/min.

Quantitative analysis was performed on a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source in the positive ionization mode. The MS operating conditions were optimized as follows: spray voltage, 4,200 V; heated capillary

temperature, 320°C; sheath gas (nitrogen), 30 Arb; auxiliary gas (nitrogen), 5 Arb; collision gas (argon) pressure, 1.2 mTorr. Data acquisition was performed by Xcalibur 2.0 software. Peak integration and calibration were performed using LCquan software. Quantification was obtained by using selected reaction monitoring (SRM) mode of the transitions at m/z 302 → 287 for dehydroevodiamine and m/z 172 → 128 for metronidazole (internal standard; IS) respectively, with a scan time of 0.3 s per transition. The HPLC system consisted of a LC-10ADvp pump and a SIL-HTA Autosampler (Shimadzu, Kyoto, Japan). Chromatographic separation was carried out on a Diamonsil C18 (150 × 4.6 mm, 5 μm, Dikma) column with an EasyGuard C18 Security Guard column (8 × 4.0 mm i.d., Dikma) kept at 20°C. The mobile phase consisted of water (containing 0.3% formic acid)–acetonitrile (30:70, v/v), at a flow rate of 0.45 mL/min.

Standard preparation

Evodia rutaecarpa powder (0.2 g) was immersed with 90 mL ethanol for 1 h, followed by ultrasonic extracting (250 W, 33 kHz) for 40 min for cooling. The solution was filtered then adjusted to 100 mL with ethanol (12). The filtrate acted as a stock standard solution with an apparent concentration of 2.0 mgH/mL, where H means herb.

Standard working solutions at the concentrations of 100, 200, 500, 2,000, 5,000, 20,000 and 50,000 ngH/mL were prepared by serially diluting the stock standard solution with methanol. A 200-ng/mL working solution of IS was similarly prepared by diluting a metronidazole stock solution with methanol. Calibration standards were prepared by spiking 100 μL of the appropriate standard working solutions into 50 μL blank plasma. Quality control (QC) samples were similarly prepared at low, medium and high levels (200, 2,000 and 40,000 ngH/mL). All solutions were stored at −20°C.

Sample preparation

Rat plasma (50 μL) was mixed with 50 μL IS solution (200 ng/mL), 150 μL methanol and 100 μL acetonitrile. After vortex-mixing for 2 min, the mixture was centrifuged at 10 krpm for 5 min. The supernatant was separated out and blown to dryness with nitrogen at 40°C. Then the residue was reconstituted in 100 μL mobile phase and a 10-μL aliquot of the final testing sample was injected onto the LC–MS system for analysis.

Method validation

The method was validated according to the currently accepted United States Food and Drug Administration (FDA) bioanalytical method validation guidance.

Method linearity was evaluated by analyzing calibration standards in duplicate at each concentration level over three consecutive days. The accuracy and precision were assessed by analyzing QC samples in six replicates at three concentration levels on three validation days. The extraction recovery was evaluated at three concentration levels by comparing the analyte/IS peak area ratios obtained from six plasma samples with the analytes spiked before and after extraction. Matrix effect was evaluated by comparing the analyte/IS peak area ratios obtained from six plasma samples with the analytes spiked after extraction to those for the neat standard solutions at the same concentrations. The stability of dehydroevodiamine in rat plasma at low and high concentration levels was evaluated under a variety of storage and process conditions.

Pharmacokinetic application

Five male Sprague-Dawley rats (250 ± 20 g) were fasted for 12 h before the experiment. The rats were split into two groups to complete the crossover design for pharmacokinetic experiment with a washout period of seven days. The powder of *Evodia rutaecarpa* and *R–E* couple was suspended in 0.1% carboxymethyl cellulose sodium (CMC-Na) aqueous solution and was administered to the rats (0.18g *Evodia rutaecarpa* powder/kg body weight) by oral gavage. Blood samples (150 μL) were obtained from the oculi chorioideae vein before dosing and subsequently at 10, 20, 45, 90, 150, 180, 210, 300, 420, 480, 720 and 1,440 min following administration, transferred to a heparinized eppendorf tube and centrifuged at 12 krpm for 5 min. The supernatants were frozen at −20°C until analysis. Pharmacokinetic parameters were calculated using Drug and Statistics 2.0 (DAS 2.0) (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Results and Discussion

Quality analysis

LC–MS^n was used for the identification of dehydroevodiamine. Through a full-scan ESI mass spectrum in the positive ion mode, dehydroevodiamine was found in the *Evodia rutaecarpa* extract, plasma samples after administration of *Evodia rutaecarpa* and plasma samples after administration of *R–E* couple. The ion at m/z 302 corresponds to dehydroevodiamine at 8.49 min. The MS data indicated the loss of a methyl radical (m/z 287) in MS^2 and then the loss of 14Da (m/z 273) in MS^3, which were identical to the MS^n spectra in literature (13). The chemical structures of dehydroevodiamine and IS are shown in Figure 1. The typical

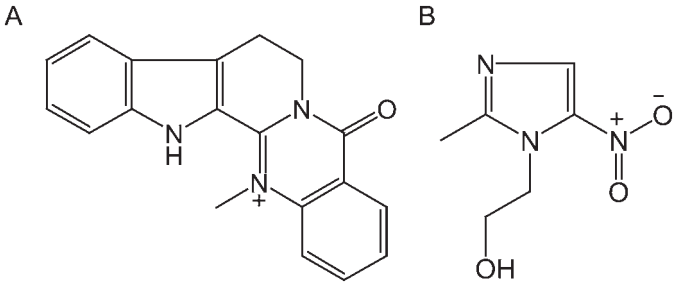

Figure 1. Chemical structures of dehydroevodiamine (A); metronidazole (IS) (B).

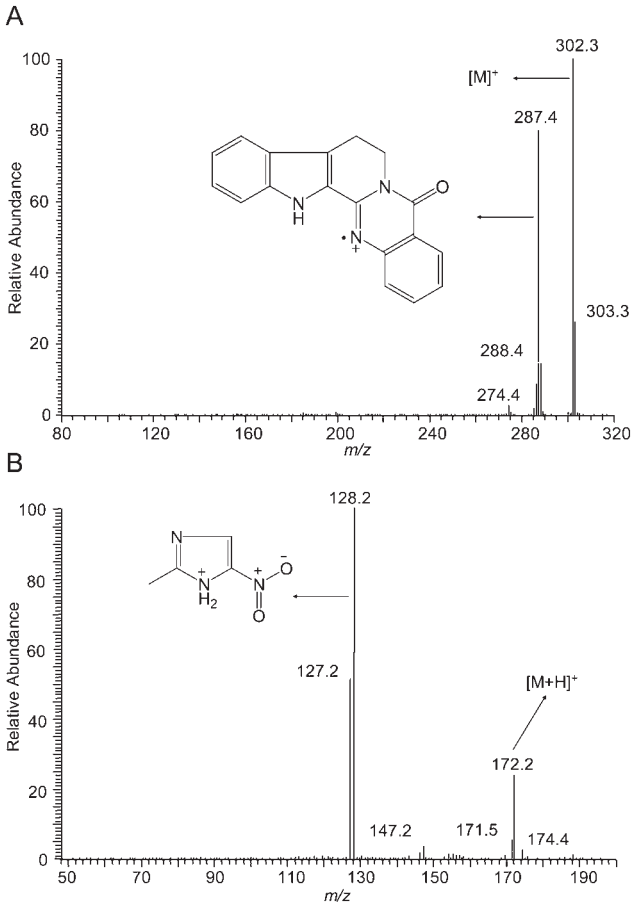

Figure 2. Product ion mass spectra of $[M]^+$ ions of dehydroevodiamine (A); metronidazole (IS) (B).

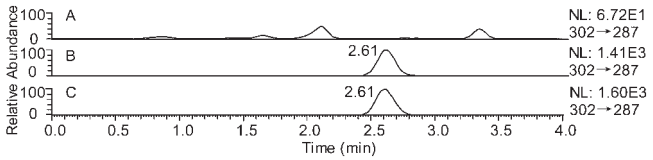

Figure 3. Representative SRM chromatograms for dehydroevodiamine (100.0 ngH/mL) in extract solution of *Rhizoma coptidis* (A); *Evodia rutaecarpa* (B); *R–E* couple (C).

full-scan ESI mass spectra of analytes and IS were described in Figure 2.

Quantity analysis

Method validation

Different herb compositions were extracted according to the procedures described previously. Figure 3 demonstrates that the components in *Rhizoma coptidis* did not affect the response of dehydroevodiamine. The typical chromatograms of a blank, a spiked plasma sample with the reference extract (100.0 ngH/mL) and IS (200 ng/mL) and plasma obtained 180 min after oral administration of *R–E* couple are presented in Figure 4. For all samples, no interference was found at the retention times of the analyte or the IS.

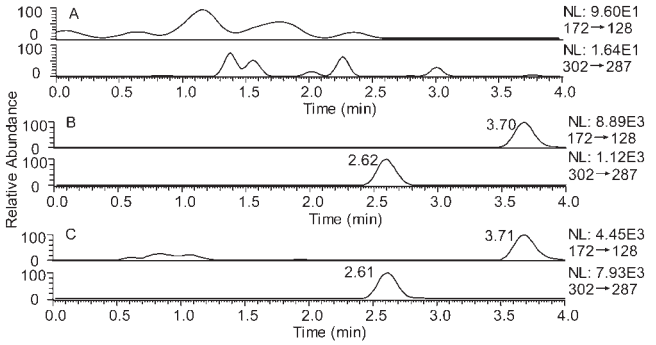

Figure 4. Representative SRM chromatograms for analytes in a blank plasma sample (A); a blank plasma spiked with standard extract at the LLOQ and IS at 200 ng/mL (B); a plasma sample 3 h after administration of *R–E* couple (C).

Table I
Intra-Day and Inter-Day Precision and Accuracy Data for Dehydroevodiamine in Rat Plasma (Three Days, Six Replicates per Day)

Added (ngH/mL)	Found (ngH/mL)	RSD%		Relative error (%)	Recovery %	
		Intra-day	Inter-day		Mean	SD
100 (LLOQ)	82.46	17.9	—	−17.5	—	—
200	180.81	14.2	13.1	−9.6	92.3	12.6
2,000	1,826.54	8.1	12.8	−8.7	91.7	7.2
40,000	34,920.42	8.8	13.8	−12.7	82.6	5.8

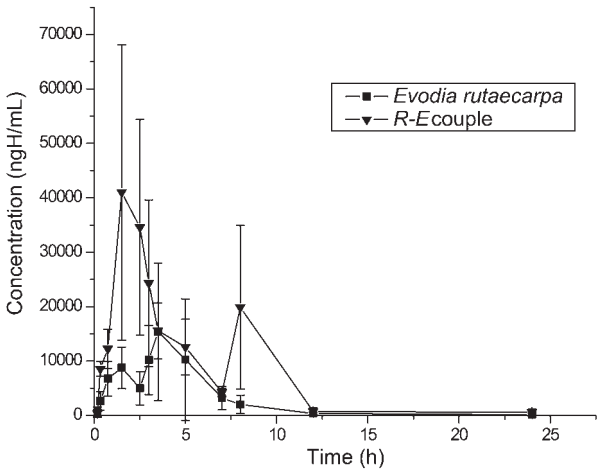

Figure 5. Mean plasma concentration–time curves of dehydroevodiamine after oral administration of *Evodia rutaecarpa* and *R–E* couple powders.

Typical equation of the calibration curve using weighted $(1/x^2)$ least squares linear regression was $Y = -6.496 \times 10^{-3} + 8.283 \times 10^{-5} X$, $r^2 = 0.9804$; over the range 100.0 ~ 50,000.0 ngH/mL. The calibration curves showed excellent linearity in rat plasma. The precision and accuracy data corresponding to lower limit of quantification (LLOQ) for dehydroevodiamine were shown in Table I. The values of accuracy and precision were within recommended limits. The extraction recoveries determined for dehydroevodiamine were shown

Table II
Mean Pharmacokinetic Parameters of Dehydroevodiamine in Rat Plasma (n = 5)

Formula	AUC_{0-t} (μgH/L/h)	$AUC_{0-\infty}$ (μgH/L/h)	MRT_{0-t} (h)	$MRT_{0-\infty}$ (h)	$t_{1/2z}$ (h)	t_{max} (h)	C_{max} (μgH/L)
Evodia rutaecarpa	68,130 ± 17,451	68,134 ± 19,162	4.6 ± 0.7	4.6 ± 5.6	1.6 ± 6.4	3.5 ± 3.0	15,383 ± 7,166
Zuojinwan	186,698 ± 46,442	186,715 ± 3,9211	4.9 ± 0.7	4.9 ± 3.6	1.8 ± 5.4	1.5 ± 1.1	40,992 ± 21,052

in Table I with acceptable variance. The matrix effect at the concentration of 2,000 ngH/mL for dehydroevodiamine and 200 ng/mL for IS in six different sources of rat plasma were 113.4 ± 5.9%. The ionization suppression/enhancement was negligible.

The stability study showed that dehydroevodiamine was stable in plasma at room temperature (25°C) for 4 h (relative error; RE < 10.7%), after three freeze–thaw cycles (RE < 8.1%) and after the reconstitution at 25° for 24 h (RE < 0.5%).

Results of pharmacokinetic study

The method described previously was applied to a pharmacokinetic study in which plasma concentrations of dehydroevodiamine were determined for 24 h after oral administration of *Evodia rutaecarpa* and *R–E* couple (0.18g *Evodia rutaecarpa* powder/kg body weight). The mean plasma concentration–time profiles ($n = 5$) are shown in Figure 5.

Pharmacokinetic parameters are listed in Table II. According to definitions, the pharmacokinetic parameters except AUC and C_{max} are independent of absolute plasma concentrations and therefore independent of standards. AUC and C_{max} are not comparable between different analytes but comparable between the same analyte in different samples.

The plasma concentration of dehydroevodiamine after oral administration of *R–E* couple was much higher than that after oral administration of *Evodia rutaecarpa* alone. Apparently, *Rhizoma coptidis* can enhance the absorption and AUC of *Evodia rutaecarpa*.

The appearance of multiple blood concentration peaks in Figure 5 is likely because the absorption rate of alkaloids was limited by their dissolution rate from herb powders. It would take more time for the constituents to dissolve out of a deep layer than a surface layer of herb powders. In addition, distribution re-absorption and enterohepatic circulation might contribute to multiple blood concentration peaks of dehydroevodiamine after oral administration of *R–E* couple.

Conclusions

In this study, dehydroevodiamine was tentatively identified by multi-stage tandem mass spectrometry. An LC–MS-MS assay was developed for the determination of dehydroevodiamine in rat plasma using the herb extract as the reference substance. The LC–MS-MS assay was applied to the comparative pharmacokinetic studies of dehydroevodiamine in plasma. This approach is well suited for the determination of the compounds without standards.

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