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Simultaneous determination of evodiamine and rutecarpine in rabbit plasma by LC-ESI-MS and its application to pharmacokinetics

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A sensitive and selective liquid chromatography-mass spectrometry (LC–MS) method for the determination of evodiamine and rutecarpine in rabbit plasma was developed and validated. The analytes and internal standard (IS) are extracted from plasma by one-step protein precipitation with acetonitrile, and separated on a Zorbax SB-C18 column (2.1 mm × 50 mm, 3.5 μm) using acetonitrile-0.1% formic acid as mobile phase with gradient elution. Electrospray ionization (ESI) source was applied and operated in positive ion mode, and selective ion monitoring (SIM) mode used to quantify evodiamine and rutecarpine. The assay is linear over the range of 2–1600 ng/mL for evodiamine and rutecarpine, with a lower limit of quantification (LLOQ) of 5 ng/mL both for evodiamine and rutecarpine. Intra-day and inter-day precision are less than 12% and the accuracy are in the range of 90.9–104.3%. Furthermore, the newly developed method is successfully used for the determination of evodiamine and rutecarpine in rabbit plasma for pharmacokinetic study.

1. Introduction

Evodiamine and rutecarpine are two naturally occurring indole alkaloids (Fig. 1) which are the main bioactive alkaloid components in the fruit of Evodiae fructus (Wen et al. 2006). It has been reported that evodiamine exerts several pharmacological effects (Liu et al. 2010), namely, anti-tumor activities (Kan et al. 2007; Lee et al. 2006), antiobesity effects (Wang et al. 2008), protection against myocardial ischemia-reperfusion injury (Rang et al. 2004), inhibition of adipogenesis (Wang et al. 2009), and that rutecarpine can induce CYP1A1 expression (Han et al. 2009). A pharmacokinetics study of evodiamine and rutecarpine could be helpful for the reasonable use of Evodia rutaecarpa and its preparations (Zhao et al. 2010). Therefore, an analytical method for the simultaneous determination of evodiamine and rutecarpine in biological samples is needed for plasma concentration monitoring.

Several methods been developed for simultaneous determination of evodiamine and rutecarpine in biological samples, respectively, such as HPLC (Yin et al. 2009), LC-MS/MS (Wen et al. 2006; Zhao et al. 2010). These reported HPLC methods suffer from selective and low sensitivity. Recently, Wen et al. (2006) reported an LC-MS-MS method for the simultaneous determination of evodiamine and rutaecarpine in human serum with a LLOQ of 5.2 and 10.2 ng/mL, respectively. However, the method suffers from the large sample volume (1 mL) and long analysis time (17 min). Zhao et al. (2010) developed a sensitive UPLC-MS-MS method for the simultaneous determination of evodiamine and rutaecarpine in rat plasma using a sample volume of 200 μL, both with a LLOQ of 0.2 ng/mL and with liquid–liquid extraction with ether used for sample preparation. In this paper, a simple and sensitive LC-MS/MS method for the simultaneous determination of evodiamine and rutecarpine in rabbit plasma using one-step protein precipitation was developed and validated. The LC-MS/MS method was successfully applied to a pharmacokinetic study of evodiamine and rutecarpine after intravenous administration to rabbits.

2. Investigations, results and discussion

2.1. Method development

Electrospray ionization (ESI+) source was used in this work, because it exhibited more sensitivity and better reproducibility for evodiamine and rutecarpine than an atmospheric pressure chemical ionization (APCI+) interface. It was amazing that the sensitivity of the SIM mode was much higher than the multiple reaction monitoring (MRM) mode with *S/N* 8/1 for SIM mode and 2/1 for MRM mode at the concentration of LLOQ (5 ng/mL). Therefore, we chose the SIM mode as the quantification mode in this study.

The liquid chromatographic conditions were optimized to separate as many interference compounds as possible from the analytes. Different columns, such as Zorbax SB-C18 (50 mm × 2.1 mm, 3.5 μm) and Zorbax Extend-C18 (50 mm × 2.1 mm, 3.5 μm) were investigated. A Zorbax SB-C18 column from Agilent demonstrated better selectivity and proper retention than Zorbax Extend-C18. Different mobile phase compositions (such as acetonitrile/methanol-ammonium acetate, acetonitrile/methanol-water, acetonitrile/methanol-0.1% formic acid) were assessed to increase the sensitivity and to obtain better and sharper peaks. Acetonitrile was chosen as the organic solvent because acetonitrile led to a sharper peak shape and lower pressure than methanol. The introduction of formic acid into the mobile phase could improve the sensitivity, therefore acetonitrile-0.1% formic acid was chosen as the mobile phase. Gradient elution provided better peak symmetry and avoided the matrix effects for the analytes and IS compared

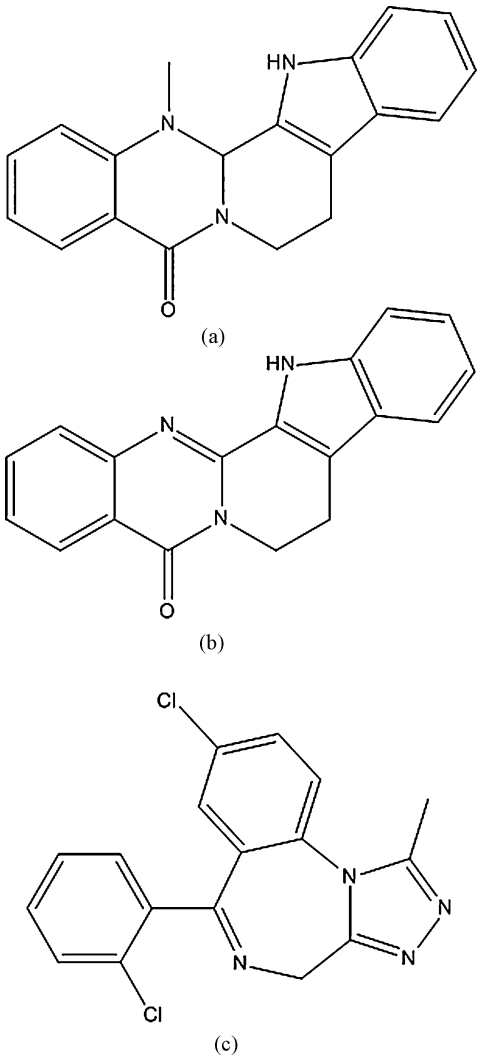

Fig. 1: Chemical structures of of evodiamine (a), rutecarpine (b) and IS (c)

to isocratic elution (Lin et al. 2011; Ma et al. 2011; Yang et al. 2011).

An efficient clean-up for bio-samples to remove protein and potential interferences prior to LC-MS analysis was an important point in the studies. In the literature, several extraction procedures for evodiamine and rutecarpine in biological samples have been employed, including liquid–liquid extraction (LLE) (Yin et al. 2009; Zhao et al. 2010) and solid-phase extraction (Wen et al. 2006). But LLE and SPE methods were tedious and time-consuming. Simple and effective protein precipitation was employed in our work, which could provide acceptable recoveries. Acetonitrile was chosen as the protein precipitation solvent because it exhibited better effect than methanol.

The internal standard (IS) was also chosen during the process of method development. To find a suitable IS, several compounds (including lidocaine, carbamazepine, bupivacaine, estazolam, triazolam, lobeline and diazepam) were tested. Triazolam was optimal because of its stable ionization in positive-ion ESI mode and suitable retention time.

2.2. Selectivity and matrix effect

Fig. 2 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with evodiamine, rutecarpine and IS, and a plasma sample. No interfering endogenous substance was observed at the retention times of the analytes and IS.

The matrix effect (ME) for evodiamine and rutecarpine at concentrations of 5, 100, and 1200 ng/mL was measured to be 94.1, 93.2 and 96.1% for evodiamine and 92.7, 91.2 and 95.2% for rutecarpine ($n = 6$), respectively. The ME for IS (200 ng/mL) was 97.2% ($n = 6$). As a result, ME from plasma was ignored in this method.

2.3. Calibration curve and sensitivity

Typical equations for the calibration curves for evodiamine and rutecarpine were: $y = 0.004x + 0.016$, $r = 0.9987$; $y = 0.0043x + 0.0474$, $r = 0.9967$. Where y represents the ratios of analyte peak area to that of IS and x represents the plasma concentration.

The lower limit of quantification (LLOQ) for the determination of evodiamine and rutecarpine in plasma both was 5 ng/mL. The precision and accuracy at LLOQ were 13.5% and 87.5% for evodiamine, 10.3% and 106.5% for rutecarpine, respectively. The limit of detection (LOD), defined as a signal-noise ratio of 3, was 1.5 and 1 ng/mL for evodiamine and rutecarpine in plasma, respectively.

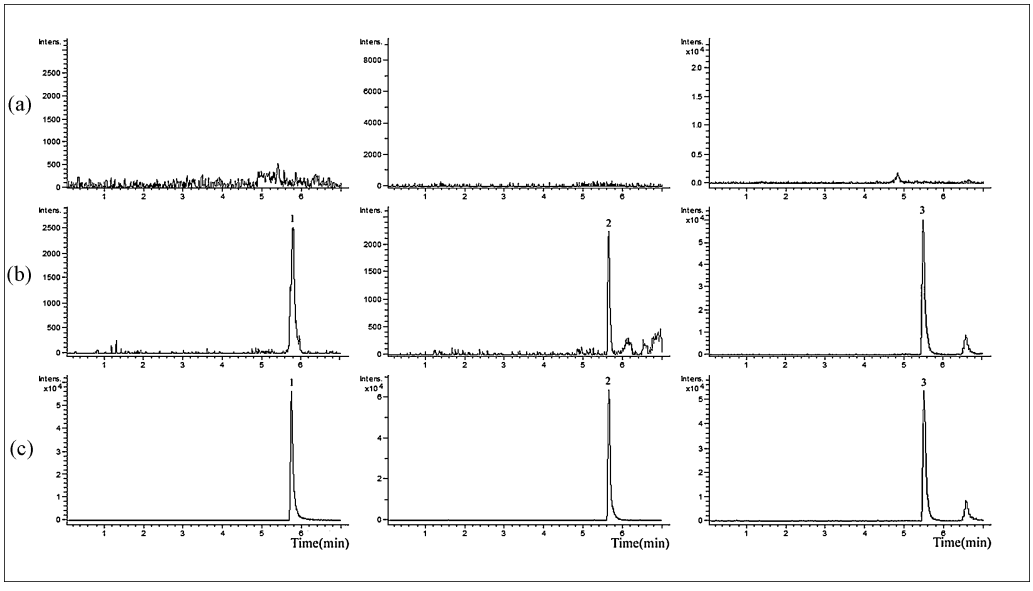

Fig. 2: Representative LC-ESI-MS chromatograms of evodiamine and rutecarpine and IS: (a) blank plasma; (b) blank plasma spiked with evodiamine (10 ng/mL) and rutecarpine (10 ng/mL) and IS (200 ng/mL); (c) a rabbit plasma sample 45 min after intravenous administration of 4 mg/kg evodiamine and 4 mg/kg rutecarpine

Table 1: Precision, accuracy and recovery for evodiamine and rutecarpine in rabbit plasma ($n = 6$)

Compound	Concentration(ng/mL)	RSD (%)		RE (%)		Recovery
		Intra-day	Inter-day	Intra-day	Inter-day	(%)
Evodiamine	5	10.2	9.8	–9.1	–2.4	90.1
	100	6.5	7.6	–5.4	2.1	94.3
	1200	5.4	7.5	4.3	–3.1	95.4
Rutecarpine	5	11.2	9.5	1.2	–2.8	92.4
	100	6.4	5.4	–4.5	–3.5	94.8
	1200	6.8	6.4	3.2	2.1	97.4

2.4. Precision, accuracy and recovery

The precision of the method was determined by calculating relative standard deviation (RSD) for quality control samples (QCs) at three concentration levels over three validation days. Intra-day and inter-day precision was 11% or less for evodiamine and 12% or less for rutecarpine. The accuracy of the method ranged from 90.9% to 104.3% for evodiamine and 95.5% to 103.2% for rutecarpine. Mean recoveries of evodiamine and rutecarpine were better than 90.1%. The recovery of the IS (200 ng/mL) was 95.2%.

Assay performance data are presented in Table 1. These results demonstrate that the method is accurate and precise.

2.5. Stability

The stability results of the auto-sampler, freeze-thaw and long-term (30 days) indicated that evodiamine, rutecarpine and IS were stable under the storage conditions described above since the bias in concentration were within ±15% of their nominal values, Table 2.

2.6. Application

The method was applied to a pharmacokinetic study in rabbits. The main pharmacokinetic parameters for two-compartment model were summarized in Table 3.

Table 2: Summary of stability of evodiamine and rutecarpine under various storage conditions ($n = 3$)

Conditions	Concentration (ng/mL)		RSD (%)	RE (%)
	Added	Found		
Evodiamine				
–20 °C, 30 days	5	4.5	9.7	–10.0
	100	103.6	6.9	3.6
	1200	1160.3	7.2	–3.3
3 freeze thaw	5	5.2	10.5	4.0
	100	94.6	9.3	–5.4
	1200	1223.6	8.5	2.0
Autosampler ambient 12 h	5	5.1	5.1	2.0
	100	98.5	2.1	–1.5
	1200	1206.8	2.3	0.6
Rutecarpine				
–20 °C, 30 days	5	5.3	8.6	6.0
	100	108.4	5.6	8.4
	1200	1158.6	6.7	–3.5
3 freeze thaw	5	4.6	10.2	–8.0
	100	107.4	7.6	7.4
	1200	1160.3	7.8	–3.3
Autosampler ambient 12 h	5	5.1	4.3	2.0
	100	98.4	3.2	–1.6
	1200	1205.9	2.4	0.5

3. Experimental

3.1. Chemicals and reagents

Evodiamine (purity > 98.0%) and rutecarpine (purity > 98.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Triazolam (purity > 98.0%), was obtained from Institute of Forensic Science under the Ministry of Justice (Shanghai, China). LC-grade acetonitrile was from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, USA). Ultra-pure water prepared by a Milli-Q purification system from Millipore (Bedford, USA).

3.2. Preparation of standard solutions

Stock solutions of evodiamine (1.0 mg/mL), rutecarpine (1.0 mg/mL) and IS (100 μg/mL) were separately prepared in 10-mL volumetric flasks with acetonitrile–water (50:50, v/v) and stored at 4 °C. Working solutions for calibration and controls were prepared from the stock solution by dilution with acetonitrile–water (50:50, v/v). The IS working solution (2.0 μg/mL) was prepared by diluting its stock solution with acetonitrile–water (50:50, v/v). Calibration curves were prepared using blank plasma spiked at concentrations of 2, 5, 10, 20, 50, 100, 200, 500, 1000, 1600 ng/mL for evodiamine and rutecarpine. Low, medium, and high quality control (QC) samples at concentrations of 5, 100, and 1200 ng/mL were prepared in a same way as the calibration standards.

3.3. Instrumentation and conditions

The LC–MS system consisted of a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version *B.01.03* [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on a 50 mm × 2.1 mm, 3.5 μm particle, Agilent Zorbax SB-C18 column at 25 °C. The flow rate was 0.3 mL/min. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0–1.5 min (10–80% B), 1.5–6.0 min (80–80% B), 6.0–7.0 min (80–10% B), 7.0–10.0 min (10–10% B).

Analyses were performed with the ESI source operated in positive mode with a drying gas (N_2) flow of 6 L/min, nebulizer pressure of 20 psi, drying gas temperature of 350 °C, capillary voltage of 3.5 kV. SIM mode was applied to quantify analyzes using target ions at *m/z* 304 for evodiamine, *m/z* 288 for rutecarpine, *m/z* 343 for IS.

3.4. Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 μL of the internal standard working solution (2.0 μg/mL) was added to 100 μL of collected plasma sample followed by the addition of 200 μL acetonitrile. The tubes were vortex mixed for 0.5 min. After centrifugation at 15,000 rmp for 10 min, the supernatant (10 μL) was injected into the LC-ESI-MS system for analysis.

3.5. Method validation

The selectivity of the method was evaluated by analyzing blank rabbit plasma, blank plasma spiked evodiamine, rutecarpine and IS, and a rabbit plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of analytes to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the concentration squared ($1/x$) in the concentration range of 2–1600 ng/mL. The LLOQ was estimated

Table 3: The main pharmacokinetic parameters after intravenous administration evodiamine and rutecarpine in 6 rabbits

Pharmacokinetic parameters	Evodiamine(Mean± SD)	Rutecarpine (Mean± SD)
$t_{1/2}$(min)	127.42 ± 23.05	257.70 ± 133.70
$MRT_{(0\text{-}t)}$ (min)	44.98 ± 11.54	37.86 ± 6.08
$MRT_{(0\text{-}\infty)}$ (min)	52.62 ± 11.23	70.28 ± 28.17
CL(L/min)	0.14 ± 0.02	0.15 ± 0.04
C_{max}(ng/mL)	877.0 ± 96.6	897.3 ± 258.1
$AUC_{(0\text{-}t)}$ (min ng/mL)	29768.25 ± 4028.81	26602.38 ± 6478.69
$AUC_{(0\text{-}\infty)}$ (min ng/mL)	30137.78 ± 4050.57	27584.40 ± 6366.84

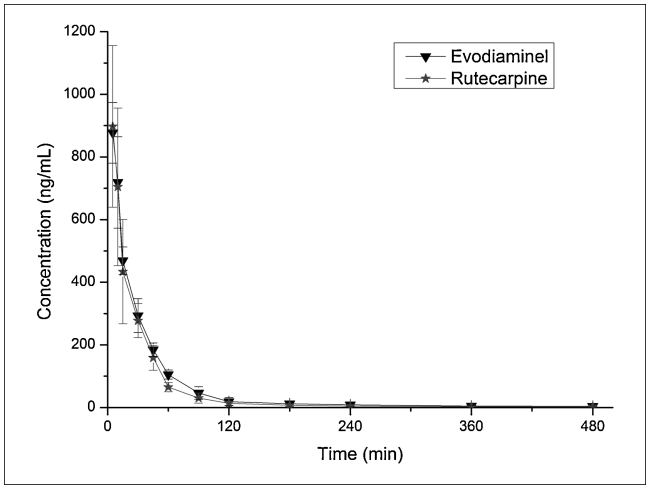

Fig. 3: Mean plasma concentration time profile after intravenous administration of 4 mg/kg evodiamine and 4 mg/kg rutecarpine in 6 rabbits

in the process of calibration curve construction and was defined as the lowest concentration for which precision (RSD) was better than 20%.

To evaluate the matrix effect, blank rabbit plasma were protein precipitated and then spiked with the analyte at 5, 100, and 1200 ng/mL (six different sources). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect (ME). The matrix effect of IS was evaluated at the working concentration (200 ng/mL) in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (5, 100, and 1200 ng/mL) in three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE).

The recoveries of evodiamine and rutecarpine at three QC levels ($n = 6$) were determined by comparing peak-area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

The stabilities of evodiamine and rutecarpine in rabbit plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 5, 100, and 1200 ng/mL, which were exposed to different conditions (Deng et al. 2009). These results were compared with those obtained for freshly prepared plasma samples. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at –20 °C for 30 days. The stability of the IS (200 ng/mL) was evaluated in a similar way.

3.6. Pharmacokinetic study

Japanese male rabbits (2.1–2.3 kg) were raised from Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China). All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Rabbits were intravenously given evodiamine and rutecarpine via a marginal ear vein at a dose of 4 mg/kg within 1 min, respectively. Blood samples (0.3 mL) were collected from the marginal ear vein into heparinized 1.5 mL polythene tubes at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480 min after dosing. The samples were immediately centrifuged at 5000 rpm for 5 min. The plasma obtained (100 μL) was stored at –20 °C until analysis. Plasma evodiamine and rutecarpine concentration versus time data for each rabbit were analyzed by DAS software (Version 2.0, Wenzhou Medical College, China). Fig. 3

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