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Application of a sensitive and specific LC–MS/MS method for determination of chinensinaphthol methyl ether in rat plasma for a bioavailability study

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ABSTRACT

Chinensinaphthol methyl ether (CME) is a potential pharmacologically active ingredient isolated from the dried plants of *Justicia procumbens L. (Acanthaceae)*. A sensitive and specific LC–MS/MS method was developed and validated for the analysis of CME in rat plasma using buspirone as the internal standard (IS). The analyte was extracted with ethyl acetate and chromatographed on a reverse-phase Agilent Zorbax-C18 110 Å column (50 mm × 2.1 mm, 3.5 μm). Elution was achieved with a gradient mobile phase consisting of water and acetonitrile both containing 0.1% formic acid at a flow rate of 0.40 mL/min. The analytes were monitored by tandem–mass spectrometry with positive electrospray ionization. The precursor/product transitions (*m*/*z*) in the positive ion mode were 394.5 → 346.0 and 386.1 → 122.0 for CME and IS, respectively. The assay was shown to be linear over the range of 0.50–500 ng/mL, with a lower limit of quantification of 0.50 ng/mL. The method was shown to be reproducible and reliable with the inter- and intra-day accuracy and precision were within ±15%. The assay has been successfully used for pharmacokinetic evaluation of CME after intravenous and oral administration of 1.80 mg/kg CME in rats. The oral absolute bioavailability (F) of CME was estimated to be 3.2 ± 0.2% with an elimination half-life ($t_{1/2}$) value of 2.4 ± 0.8 h, suggesting its poor absorption and/or strong metabolism *in vivo*.

1. Introduction

Justicia procumbens L. (*Acanthaceae*), known as "Juechuang" in China, is a notable traditional Chinese medicines, which widely distributes in south and southwest China and adapts well to tropic and subtropical climate. The whole plant of *Justicia procumbens L.* (*Acanthaceae*) is used as herbal remedy for the treatment of fever, pain due to laryngopharyngeal swelling and cancer in China [1,2]. In recent years, extensive phytochemical and pharmacological studies have been performed to isolate the family of lignans and their glycosides in our laboratory [3–8]. Chinensinaphthol methyl ether (CME, Fig. 1), a major pharmacological active ingredient, is one of the most abundant components in *Justicia procumbens*.

Bioassay results revealed that CME exhibited antitumor [4,7,9], anti-platelet [5,10] and antiviral activities [8]. In the on-going studies, CME was selected as one of the chemical markers for quality control of *Justicia procumbens* raw material [11]. Furthermore, due to its potential antitumor effects, CME is now being further investigated as a new drug-development lead compound.

CME in *Justicia procumbens* and its compound preparation Jianer syrup was unequivocally identified with standard by HPLC-UV fingerprints for the quality evaluation [12,13]. However, these methods do not meet the analytical requirements for biological fluids with respect to an efficient clean-up procedure, shorter runtime and higher sensitivity. To the best of our knowledge, there are no published analytical methods available for the quantification of CME in biological fluids and its pharmacokinetic profile has not been investigated till now.

Therefore, the purpose of this study was to develop a sensitive and specific LC–MS/MS method suitable for the quantification of CME in rat plasma. The method has been successfully applied to the pharmacokinetic evaluation of CME using the rat as an animal model.

2. Materials and methods

2.1. Chemicals and reagents

Reference standard of CME (Free base, Batch No. 20110923) was isolated from *Justicia procumbens* and identified by UV, IR, ESI-MS, ^{1}H- and ^{13}C-NMR [14]. Purity of CME was higher than 98.3% by normalization of the peak areas detected by HPLC-UV at 256 nm [13]. Buspirone (Internal standard, IS) was purchased

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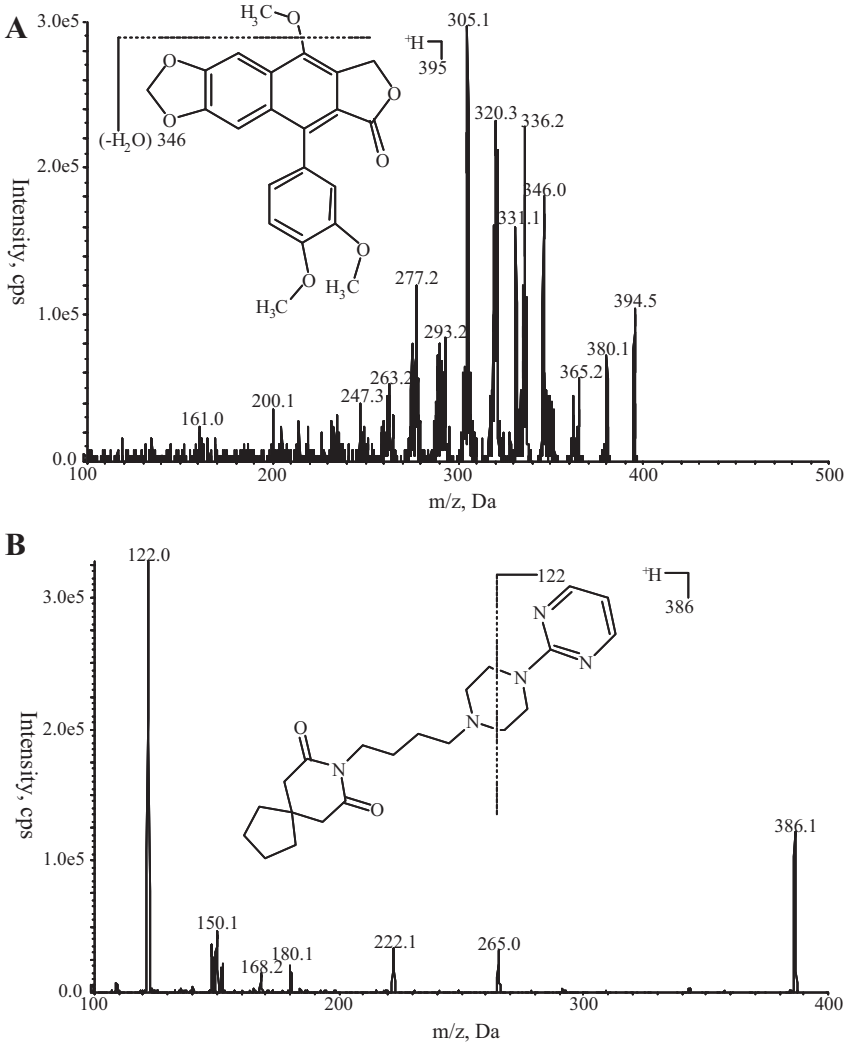

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation schemes for (A) CME and (B) buspirone (internal standard).

from the Sigma Chemical Co. (ST. Louis, MO, USA). Sulfobutylether-beta-cyclodextrin (SBECD) was purchased from Cydex (Lenexa, KS, USA). DMSO of analytical grade was purchased from TEDIA (USA). Methanol and acetonitrile of HPLC grade were obtained from Fisher Co. Ltd. (Emerson, IA, USA). Formic acid and other reagents were of analytical grade and purchased from MREDA Chemical Reagent Company (Beijing, China). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. LC–MS/MS

The HPLC system consisted of an LC-20AD pump, a DGU-20 A_3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). CME and IS were separated on a reverse phase Agilent Zorbax-C18 110 Å column (50 mm × 2.1 mm, 3.5 μm), which was eluted with a gradient mobile phase consisting of water (A) and acetonitrile (B) both containing 0.1% formic acid. A two-step gradient elution program was utilized as follows: 0.00 min 2% B, 0.60 min 2% B, 3.00 min 80% B, 5.00 min 100% B, 5.50 min 100% B, 7.00 min 2% B. The flow rate was set at 0.40 mL/min.

The column effluent was monitored using a 4000 QTRAP® LC/MS/MS (AB Sciex, Toronto, Canada). The ESI source was operated in positive mode with the curtain, nebulizer and turbo-gas set at 12, 60 and 60 psi, respectively. The curtain, nebulizer, heater and collision gases were all nitrogen. The turbo-gas temperature was 550 °C and the ion spray needle voltage was 5500 V. Optimal multiple reaction monitoring (MRM) was used to detect transition ions from a specific precursor ion to product ion for CME ($[M+H]^+$ m/z 394.5 → 346.0) and the internal standard ($[M+H]^+$ m/z 386.1 → 122.0). The collision energy was set at 33 and 45 eV for CME and the internal standard, respectively.

2.3. Preparation of standards and calibration curves

Separate stock solutions of CME and IS (1.0 mg/mL) were prepared by dissolving appropriate amount of each reference standard in DMSO, and were refrigerated until used. A series of CME working standard solutions were prepared by appropriate dilutions of the stock CME standard solution (1.0 mg/mL) with DMSO to obtain the following CME concentrations: 5000, 2000, 1000, 500, 200, 100, 50, 20, 10 and 5.0 ng/mL. These standard solutions of CME were used to spike blank rat plasma with CME to yield calibration standards in plasma over the concentration range of 0.50–500 ng/mL. Briefly, the CME spiking procedure involved transferring 5 μL aliquot of various CME working standard solutions and 50 μL aliquot of blank plasma into 1.1 mL centrifuge tubes. QC (quality control) samples were prepared in a similar manner at low, medium and high CME levels (1.0, 50, 400 ng/mL). The IS (Buspirone) working solutions (10 ng/mL) was prepared by diluting with water. All the stock and working standard solutions were stored at 4 °C prior to use.

2.4. Sample preparation

After thaw at room temperature for about 30 min and vortex for 30 s, aliquots of 50 μL plasma were mixed with 5 μL of methanol (or standard or QC solution) and 10 μL of IS solutions and 400 μL of ethyl acetate. After vortex for 1 min and then centrifugation at 12000 *g* for 10 min, aliquots of 300 μL supernatants were removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were dissolved in 150 μL of the mixture of methanol and water (50:50, v/v), and then transferred to HPLC vials. A volume of 10 μL of this solution was then injected onto the column.

2.5. Assay validation

2.5.1. Linearity, accuracy, precision, and recovery

Linear calibration curves in rat plasma were generated by plotting the peak area ratio of CME to the IS versus the known plasma CME concentrations over the range of 0.50–500 ng/mL. Slope, intercept and coefficient of determination values were estimated using least square regression analysis. Quality control plasma samples containing low, medium, and high CME concentrations were used to evaluate the precision and accuracy of the assay method. The intra-day assay precision and accuracy were obtained by analyzing six replicates of the quality control samples in duplicate using a calibration curve constructed on the same day. The inter-day assay precision and accuracy were obtained by analyzing six quality control samples in duplicate using calibration curves constructed on 3 different days. Intra-day and inter-day precisions of the method were expressed by [(standard deviation)/(mean concentration)] × 100. Accuracy of the method was expressed by [(mean measured concentration − nominal concentration)/(nominal concentration)] × 100. The mean values and RSD for QC samples at three concentration levels were calculated using a one-way analysis of variance (ANOVA). The assay precision was reflected by the relative standard deviation (RSD%) and the assay accuracy was reflected by the relative percentage error from the theoretical drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with acceptable precision (% RSD ≤ 20%) and accuracy (% RE within ±20%). The extraction recoveries of CME from rat plasma (expressed as a percentage) were calculated as the ratio of the slope of a calibration curve for CME in spiked plasma to that in spiked mobile phase.

2.5.2. Stability

The stability of CME in rat plasma was investigated at three QC levels, as described in Section 2.3. Stability tests of the analyte were performed on six replicates of three QC concentrations after (a) three freeze (−20 °C) and thaw cycles, (b) reconstituted extract at 4 °C for 24 h and (c) stored at −80 °C for a month, respectively.

2.5.3. Matrix effects

Matrix effects from endogenous substances present in extracted rat plasma may cause ion suppression or enhancement of the signal. Matrix effects were assessed by comparing the peak areas of CME after addition of low ($n = 3$) and high ($n = 3$) concentrations of CME to (A) mobile phase and (B) the supernatant of extracted blank plasma. These studies were conducted with six different lots of rat plasma. The peak area ratio of B/A (as a percentage) or the percentage matrix factor was used as a quantitative measure of the matrix effect.

2.6. Pharmacokinetic study

The protocols of this animal study were approved by Animal Care and Use Committee, Chinese Academy of Medical Science and Peking Union Medical College. Six male SD rats weighing 200–250 g were purchased from Beijing Military Medical Sciences Experimental Animal Co., Ltd. (Beijing, China). The rats were certified and had not been dosed with any pharmaceutical before the experiment. The rats were housed under standard conditions and had *ad libitum* access to water and a standard laboratory diet. Polyethylene cannulas were implanted in the femoral vein 2 days before the experiment while the rats were anesthetized using isoflurane inhalation. The cannulas were externalized at the back of the neck and filled with heparinized saline (20 units/mL) to prevent blood clotting.

Each rat was housed individually in a rat metabolic cage and was not restrained at any time during the study. The rats were fasted for 16 h before experiments with the exception of free access to water. The dosing solution with CME concentration of 0.36 mg/mL was prepared by dissolving appropriate amount of CME in DMSO:30% SBECD (5:95, v/v). The actual intravenous and oral doses of CME were both 1.80 mg/kg, which was consistent with the medium dose level used in the pharmacological experiments, and the dose volume was 5.0 mL/kg. After intravenous administration of 1.80 mg/kg CME through tail vein, aliquots of 0.20 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.033, 0.083, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 h). After oral administration, aliquots of 0.20 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.25, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 h). Heparinized blood was centrifuged at 12000 × *g* at room temperature for 5 min to obtain plasma, which was stored at −80 °C until analysis.

Pharmacokinetic parameters including half-life ($t_{1/2}$), maximum plasma time (t_{max}) and concentration (C_{max}), area under concentration–time curve (AUC_{last} and AUC_{Inf}), clearance (CL), steady-state volume of distribution (V_{ss}), mean residence time (MRT) of CME were analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, USA). All results were expressed as arithmetic mean ± standard deviation (SD).

3. Results

3.1. Chromatography

This study first describes the development of a sensitive and specific LC–MS/MS assay for the determination of CME concentrations in rat plasma. The full-scan product ion mass spectra of CME and buspirone (Internal standard) are shown in Fig. 1. Mass chromatograms of CME and IS obtained by extraction of blank rat plasma, blank plasma spiked with CME and IS, and actual unknown plasma samples obtained in rats after intravenous and oral injection of CME (dose 1.80 mg/kg) are shown in Fig. 2. The chromatographic run time for the extracted plasma samples was 7.0 min. The retention times for CME and IS were 4.29 and 3.39 min, respectively. The chromatograms show baseline separation of CME and the internal standard without any interference from endogenous plasma components.

3.2. Linearity, sensitivity and detection limit of the assay

Calibration standards were prepared by spiking 5 μL of the appropriate standard solutions of CME to 50 μL of blank rat plasma. Plasma concentrations were 0.50, 1.0, 2.0, 10, 50, 100, 200 and 500 ng/mL for CME. The peak area (y) and concentration of CME (x) were subjected to a weighted ($1/X^2$) least squares linear regression analysis to calculate calibration equation and correlation coefficients. The linear ranges of CME in rat plasma were from 0.50–500 ng/mL. The lower limit of quantification (LLOQ) of CME

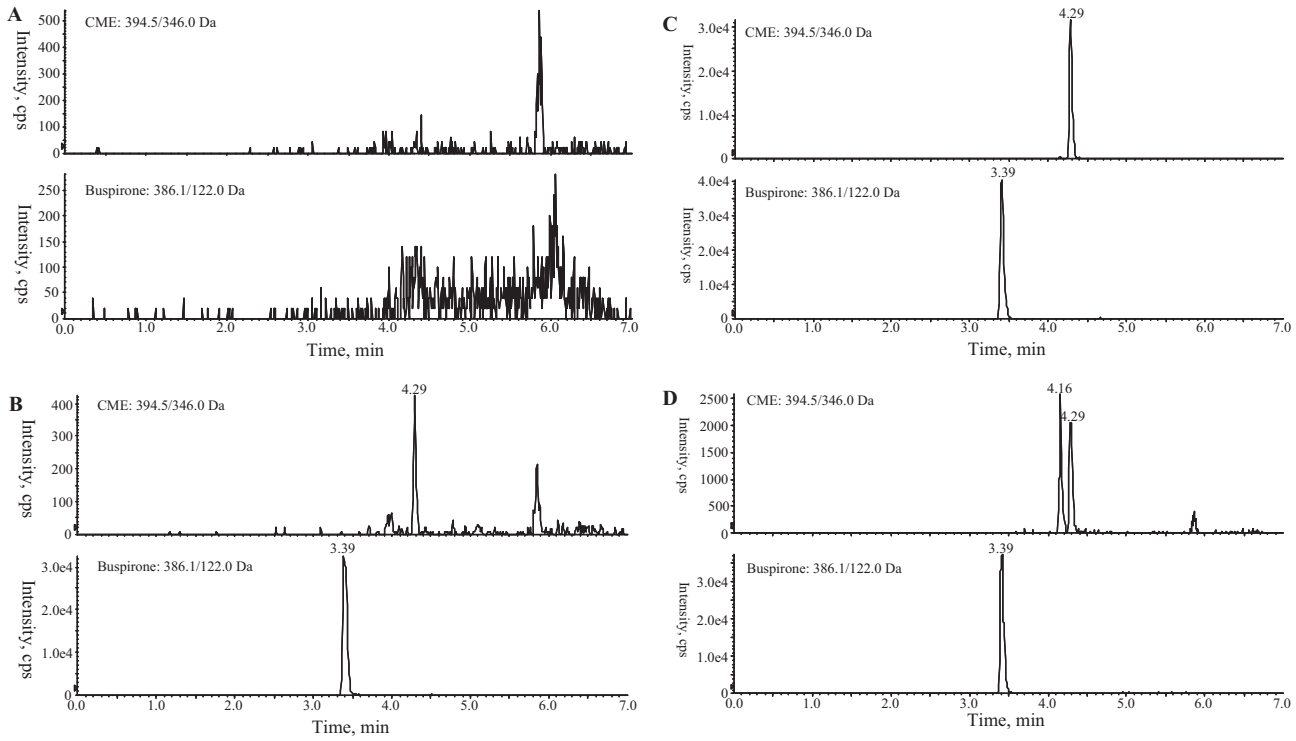

Fig. 2. Typical ion chromatograms of (A) blank rat plasma; (B) blank rat plasma spiked with CME (0.5 ng/mL, LLOQ) and IS; (C) an unknown rat plasma sample (calculated CME concentraton: 400 ng/mL, 10-fold dilution using blank rat plasma) collected at 15 min after intravenous administration of 1.80 mg/kg CME; and (D) an unknown rat plasma sample (calculated CME concentraton: 10.2 ng/mL, 3-fold dilution using blank rat plasma) collected at 30 min after oral administration of 1.80 mg/kg CME.

was 0.50 ng/mL. Typical equations for the standard curves were $y = 0.0501\,x - 0.0001$ ($r = 0.9968$).

3.3. Extraction recovery and matrix effect

The extraction recovery and matrix effect results are summarized in Table 1. The mean recoveries of CME were all above 80%. The data indicated that the recoveries of CME from rat plasma were concentration-independent in the concentration range evaluated and the recoveries were acceptable for the pharmacokinetic analysis. A mean percentage matrix effect value of 89.9% for CME was calculated and found to be independent of CME plasma concentration and rat plasma lot. This result is in agreement with international guidelines and indicates low ion suppression [15,16].

3.4. Accuracy and precision of the assay

To determine the intra-day precision of the method, three plasma samples with the concentrations of 1.0, 50 and 400 ng/mL were analyzed six times on the same day. To determine the inter-day precision and the accuracy, further three plasma samples were run on each of three different days. Table 2 summarizes the intra- and inter-day precision and accuracy for CME from QC samples in rats, respectively.

Table 1
Matrix effects and recoveries of CME in rat plasma ($n = 5$).

Spiked concentration (ng/mL)	Matrix effect (%)	Mean ± SD (%)	Recovery (%)	RSD (%)
1.00	87.8	89.9 ± 2.4	92.5	9.0
50.0	89.5		93.2	9.5
400	92.5		90.8	5.7

3.5. Stability

The described stability data are summarized in Table 3. The results indicated that CME at the three concentrations tested had acceptable stabilities after three cycles of freeze–thaw, at room temperature for 24 h and at −80 °C for 1 month with the % RE values being within ± 15%.

3.6. Application of the assay method

The analytical procedures described were used to quantify CME in the plasma samples obtained from the male SD rats which were intravenously and orally administered a single dose of 1.80 mg/kg CME. The plasma concentration–time profiles of CME in rats are shown in Fig. 3 and the main pharmacokinetic parameters of CME after intravenous and oral administration are presented in Table 4.

Table 2
Precision and accuracy of the assay method for CME in rat plasma.

Batch	No.	Low 1.0 ng/mL	Medium 50 ng/mL	High 400 ng/mL
Day 1	Mean ± SD	0.99 ± 0.0	49.8 ± 2.4	403 ± 30
	RSD (%)	3.8	4.8	7.5
	Accuracy (%)	99.0	99.6	100.8
Day 2	Mean ± SD	0.97 ± 0.0	50.2 ± 4.0	401 ± 24
	RSD (%)	3.9	7.9	6.0
	Accuracy (%)	97.0	100.4	100.3
Day 3	Mean ± SD	0.99 ± 0.0	52.2 ± 3.1	400 ± 25
	RSD (%)	4.1	5.9	6.2
	Accuracy (%)	99.0	104.4	100.0
Inter-day	Mean ± SD	0.98 ± 0.0	50.7 ± 3.2	401 ± 25
	RSD (%)	3.8	6.3	6.2
	Accuracy (%)	98.0	101.4	100.3

Table 3
Stability of CME in rat plasma ($n = 5$).

Stability conditions	Added Conc.	1.0 ng/mL	50 ng/mL	400 ng/mL
Three freeze–thaw cycles	Mean ± SD	1.02 ± 0.1	49.8 ± 5.0	396 ± 35
	RSD (%)	9.3	10.0	8.9
	Recovery (%)	101.9	99.7	99.0
Room temperature for 24 h	Mean ± SD	0.90 ± 0.05	47.5 ± 3.0	383 ± 26
	RSD (%)	5.8	6.3	6.9
	Recovery (%)	89.7	94.9	95.7
Storage at −80 °C for 1 month	Mean ± SD	0.98 ± 0.10	46.7 ± 3.6	366 ± 18
	RSD (%)	9.8	7.7	5.0
	Recovery (%)	98.5	93.4	91.5

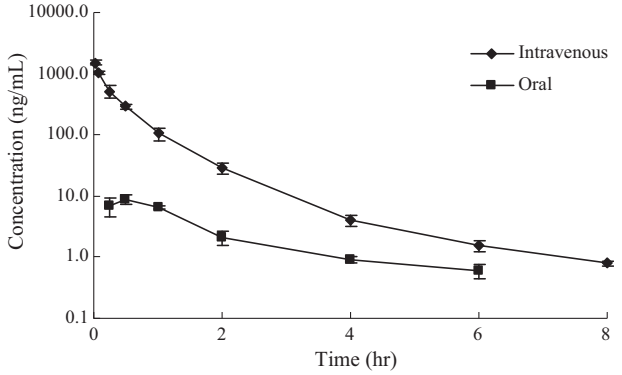

Fig. 3. Mean plasma concentration–time profiles of CME determined by LC–MS/MS method after intravenous and oral administration of 1.80 mg/kg CME to rats. Each point represents mean ± SD ($n = 3$).

4. Discussion and conclusion

This study first describes the development of a sensitive and specific LC–MS/MS assay for the determination of CME concentrations in rat plasma. In the full-scan Q1 mass spectrum, the parent positive ion peak of CME appeared at $m/z = 394.5$, and the abundance of this ion peak was sufficient for the quantification of CME. For buspirone, the most abundant peak was the protonated molecular ion $[M+H]^+$ found at $m/z = 386.1$. Thus, protonated CME and buspirone were targeted for fragmentation, and the most stable and abundant ions in the product ion scan of CME and buspirone were m/z 346.0 and 122.0, respectively. Subsequently, the mass transitions were monitored at m/z 394.5 → 346.0 for CME and m/z 386.1 → 122.0 for buspirone. Other conditions such as ion spray voltage, curtain gas pressure, nebulizer gas pressure, heater gas pressure, source temperature and collision energy were further optimized to improve the sensitivity and response stability of CME.

During the optimization of chromatographic conditions, CME was extensively retained on several kinds of columns due to its strong lipophilicity. To achieve symmetric peak shapes of CME and short chromatographic runtime, and further minimize the interferences from impurities (coexisting arylnaphthalene lignans such as 6′-hydroxy justicidin B and 6′-hydroxy justicidin C etc) and potential metabolites (through *in vivo* open-looped, cyclized and stereo-conversion), the mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used on a Zorbax C_{18} column. As shown in Fig. 2D, a interference peak at 4.16 min was found in the unknown plasma samples collected after oral administration, which was not present in the plasma samples collected after intravenous dose. It is considered to be a potential metabolite of CME *in vivo* through stereo-conversion. To separate CME from its potential stereo-isomer, a two-step gradient elution program was finally utilized as follows: 0.00 min 2% B, 0.60 min 2% B, 3.00 min 80% B, 5.00 min 100% B, 5.50 min 100% B, 7.00 min 2% B.

Because CME is insoluble in water, a mixed solvent of DMSO:30% SBECD (5:95, v/v) was adopted in order to obtain a clear solution for intravenous injection. Using the present solvent, a clear solution of 1.80 mg/mL was achieved, which was sufficient to meet the requirements of this pharmacokinetic study.

Internal standard is usually required in LC–MS/MS analysis in order to rectify the probable error in sample processing and determination. Usually an isotope-labeled internal standard is the optimal choice, however, it is difficult to obtain during the period of method development. In this study, buspirone, a readily available compound, was selected as the IS. Buspirone displays similar chromatographic retention behavior ($t_R = 3.39$ min) with CME and high extraction efficiency (>80%). In addition, there were no interferences of IS from CME and endogenous substances.

Two pretreatment methods were investigated: protein precipitation and liquid–liquid extraction. Protein precipitation provides a simple method of sample preparation and has been widely used for the analysis of analytes in plasma. After protein precipitation with methanol or acetonitrile as the protein precipitator, however, the plasma samples contained some endogenous interference that caused a rise in column pressure and distortion of peak shape. These problems were overcome using a liquid–liquid extraction method to extract the analytes of interest. The efficiencies of ethyl acetate and ethyl ether as extract solvents were evaluated, and the recoveries of CME were higher (>90%) when ethyl acetate was used as compared with ethyl ether, which yielded about a 50% recovery for CME.

In our research, it was found that CME was rapidly absorbed into the circulation system and reached its peak concentration at 30 min after oral administration. However, its absolute bioavailability was quite low with a value being 3.2%. There was significantly more apparent clearance of the CME following oral than intravenous administration, a significant first pass effect and poor permeability through the intestinal epithelial membrane after oral administration might be responsible for the low bioavailability of this compound. Our results further support efficacy results following intravenous administration.

In conclusion, the developed LC–MS/MS method for the determination of CME in rat plasma offers sufficient selectivity, accuracy and precision. The method has been successfully applied to the pharmacokinetic evaluation of intravenous and oral administration of CME using the rat as an animal model, and is currently being applied for further pharmacokinetic characterizations of CME in dogs and monkeys.

Table 4
Main pharmacokinetic parameters of CME in rats determined after intravenous and oral injection at 1.80 mg/kg CME ($n = 3$, mean ± SD).

PK Parameters	Unit	Intravenous	Oral
$t_{1/2}$	h	1.8 ± 0.2	2.4 ± 0.8
C_0	ng/mL	1974± 375	–
T_{max}	h	–	0.5 ± 0.0
C_{max}	ng/mL	–	8.7 ± 1.5
AUC_{last}	h ng/mL	528± 67.3	14.8 ± 1.8
AUC_{Inf}	h ng/mL	530± 67.2	17.0 ± 1.2
AUC_{Extr}	%	0.4 ± 0.1	13.0 ± 7.1
V_z	L/kg	8.8 ± 1.7	–
CL	mL/min/kg	57.5 ± 6.8	–
V_{ss}	L/kg	2.2 ± 0.2	–
MRT	h	0.6 ± 0.0	2.7 ± 0.8
F	%	–	3.2 ± 0.2

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