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Simultaneous determination of esculin and its metabolite esculetin in rat plasma by LC–ESI-MS/MS and its application in pharmacokinetic study

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ABSTRACT

A new liquid chromatography–tandem mass spectrometry (LC–MS/MS) method operated in the negative electrospray ionization (ESI) switching mode has been developed and validated for the simultaneous determination of esculin and its metabolite esculetin in rat plasma. After addition of internal standards scopoletin, the plasma sample was pretreated by solid-phase extraction (SPE), and separated on a reversed phase C_{18} column with a mobile phase of 0.01% formic acid in water (solvent A) and methanol (solvent B) using isocratic elution (A:B = 20:80, v/v). The detection of target compounds was done in multiple reaction monitoring (MRM) mode. The MRM detection was operated in the negative ESI mode using the transitions of *m/z* 339.1 ($[M-H]^-$) → 176.7 for esculetin, *m/z* 176.9 ($[M-H]^-$) → 133.0 and *m/z* 191.0 ($[M-H]^-$) → 175.9 for scopoletin. The standard curves, which ranged from 25 to 3200 ng/mL for esculin with the lowest limit of quantification (LLOQ) of 0.25 ng/mL and from 1.25 to 160 ng/mL for esculetin with the LLOQ of 1.25 ng/mL, were fitted to a 1/*x* weighted quadratic regression model. The method also afforded satisfactory results in terms of the sensitivity, specificity, precision (intra- and inter-day, RSD < 8.73%), accuracy, recovery as well as the stability of the analyte under various conditions. The method was successfully applied to study the pharmacokinetics of esculin and its metabolite esculetin in rat plasma after oral administration of esculin at a dose of 100 mg/kg.

1. Introduction

Cortex fraxini (Chinese name Qin-pi, CF), a commonly used Chinese herbal medicine is the dried bark of *Oleaceae* plant *Fraxinus rhynchophylla*, *F. chinensis*, *F. szaboana* and *F. stylosa*, and is mainly distributed throughout the north of China. It is officially listed in the Chinese Pharmacopoeia [1]. CF has been proven to be effective in the treatment of diarrhea and bacillary dysentery in China for over 2000 years [2–5]. Moreover, in Korea, Japan and India, CF has been demonstrated to treat arthritis and gout by enhancing uric acid excretion [6–8]. As two important coumarin derivatives, esculin and esculetin are the two major bioactive constituents in CF [9]. Esculin have multiple biological functions including intestinal anti-inflammatory activity [10], anti-oxidant activity [11] and growth inhibition of human leukemia cells and anti-cancer activity [12,13]. Esculetin is the aglycone metabolites of esculin, and it also is one of the simplest coumarins with two hydroxyl groups at carbons 6 and 7 that serve as targets for O-methylation or O-glycosylatin. Esculetin have strong antioxidative and photo-protective activities [11]. Esculetin also displays multiple immunomodulatory effects on murine lymphocytes and peritoneal macrophages in rat liver [14], including anti-inflammatory activity, inhibition of lipoxygenase and tyrosinase activity, a scavenge of hydroxyl radicals and suppressing lipid peroxidation.

Many methods, including high performance liquid chromatography with UV or fluorescence detection [15], high performance capillary electrophoresis [16,17], paper chromatography, polarography thin layer chromatography, capillary electrophoresis end-column amperometric detection [18], capillary zone electrophoresis [16] and non-aqueous capillary electrophoresis with UV detection [19,20] have been developed for determination of esculin and esculetin in plant samples or biological fluids. Spectrophotometric method including fluorescence and ultraviolet detector are currently to quantify esculin. HPLC coupled to mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) has almost completely replaced other detection systems in the field of bio-analytical research because of its high sensitivity and specificity. Therefore, it is of interest to develop a sensitive and reliable method to measure esculin and aesculentin. In this study, a sensitive and reliable high performance liquid chromatography electrospray tandem mass spectrometry (HPLC–ESI-MS/MS) method was developed for the simultaneous determination of esculin and aesculentin in rat plasma which may enrich our understanding of the complicated structure–activity relationships

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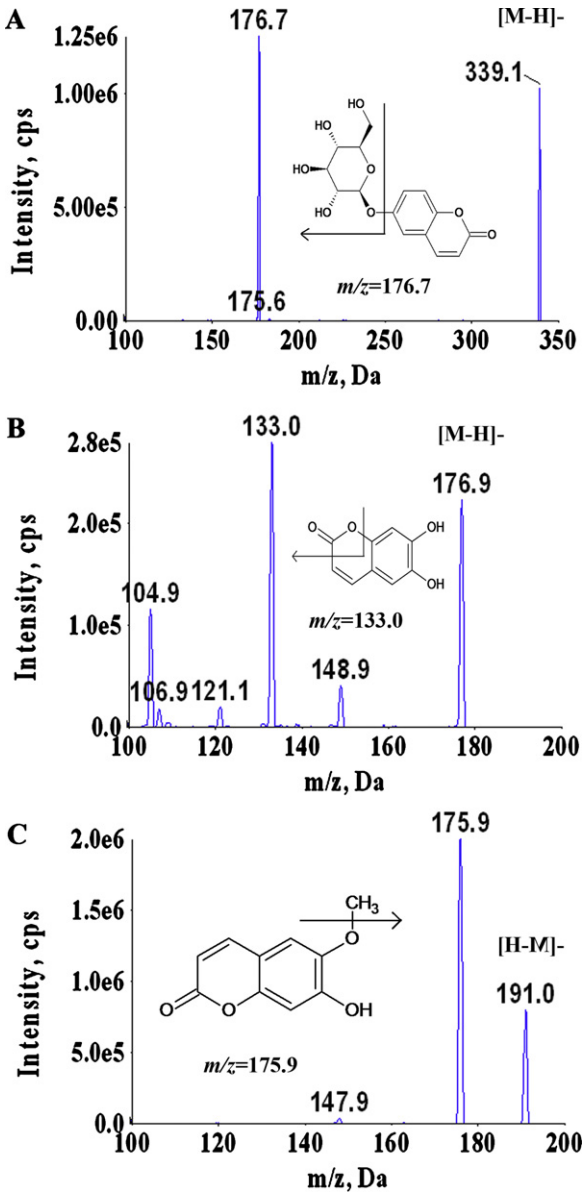

Fig. 1. MS/MS spectra of esculin (A), esculetin (B) and internal standard scopoletin (C) showing prominent precursor to product ion transitions.

between esculin and esculetin. To the authors' knowledge, this is the first report on the simultaneous determination of esculin and esculetin in rat plasma using HPLC–ESI-MS/MS.

2. Experimental

2.1. Chemicals and reagents

Esculin (>98% purity), esculetin (>98% purity) and scopoletin (>98% purity, internal standard, IS) were purchased from the Guangzhou Institute for Drug Control (Guangdong Province, China). Chemical structure of esculin, esculetin and scopoletin are shown in Fig. 1. HPLC grade methanol was purchased from Merck (Darmstadt, Germany). Formic acid was obtained from Sigma (St. Louis, MO, USA). Water was distilled and purified using a Milli-Q Plus system (Millipore, Bedford, MA, USA). All other chemicals and solvents were analytical grade and purchased from commercial sources.

Blank rat plasma was prepared in the laboratory animal center of Guangzhou University of Chinese Medicine. Freshly obtained drug free rat plasma was collected from male Sprague-Dawley rats (Medical Experimental Animal Center of Guangdong Province, Guangdong, China) in our laboratory and stored at −20 °C until the time to be used.

Table 1
Optimized mass parameters for esculin, esculetin and the internal standard.

Analyte	MRM (*m*/*z*)	DP (V)	EP (V)	CE (V)	CXP (V)
Esculin	339.1 → 176.7	−78.5	−10.5	−30.0	−8.0
Esculetin	176.9 → 133.0	−65.5	−10.0	−29.0	−12.0
Scopoletin (IS)	191.0 → 175.9	−50.6	−13.0	−18.0	−13.1

2.2. Instrumentation

Experiments were conducted using an Agilent 1200 series liquid chromatographic system consisting of a G1311A Quart pump, a G1322A degasser, a G1313A automatic sampler (ALS) and a G1316A thermostatted column compartment (TCC) (Agilent Technologies, USA) coupled to an ABAPI4000 triple quadruple mass spectrometer (Applied Biosystem/MDS-SCIEX, Foster City, CA, USA) and coupled with electrospray ionization (ESI). All evaluations were performed at unit resolution in the negative ion electrospray (ESI) mode with multiple reaction monitoring (MRM). All chromatographic separations were separated on a Gemini C_{18} 110A column (50 mm × 2.0 mm, 5 μm, Phenomenex) preceded by a guard column with the same material (4 mm × 2.0 mm, 5 μm)

2.3. Liquid chromatographic conditions

The mobile phases consisted of 0.01% formic acid in water (mobile phase A) and methanol (mobile phase B) (A:B = 20:80, v/v). Following sample injection (5 μL) into the LC–MS/MS system, analytes were separated under isocratic conditions at a flow rate of 0.3 mL/min. The cycle time of the method was 2.5 min per injection. The column temperature was kept at 40 °C.

2.4. Mass spectrometric conditions

Optimal mass spectrometer parameters employed for detection of all analytes and IS detection were as follows: ion spray source temperature at 400 °C, nebulizer gas (gas 1), turbo gas (gas 2) and curtain gas (CUR): nitrogen, 25 psi; ionspray voltage (IS) at −4500 V. Unit mass resolution was set in both mass-resolving quadruples Q1 and Q3. The detection of the ions was performed in the multiple reaction monitoring (MRM) mode. The specific parameters for each analyte are shown in Table 1. Data processing was performed on Analyst 1.4.1 software package (Applied Biosystems, MDS Sciex Toronto, Canada).

2.5. Preparation of calibration standards and quality control (QC) samples

The primary stock solutions of the analyte and IS were prepared in methanol (1 mg/mL) and stored at 4 °C and were brought to room temperature before use. The plasma calibration standards of esculin were prepared as follows: 10 μL of the working solution was evaporated to dryness by a gentle stream of nitrogen, and then 50 μL of blank rat plasma was added to obtain the concentrations of 3200, 1600, 800, 400, 200, 100, 50 and 25 ng/mL. Another set of the plasma calibration standards of esculetin was as the same way to obtain the concentrations of 160.00, 80.00, 40.00, 20.00, 10.00, 5.00, 2.50 and 1.25 ng/mL. Quality control (QC) samples were prepared in the same way as the calibration samples, representing low, medium and high concentrations of esculin and esculetin in

plasma at 50, 800, 2500 ng/mL and 2.5, 40.0, 125.0 ng/mL, respectively.

2.6. Recovery

The recovery of esculin, esculetin and IS, through solid phase extraction procedure (SPE), was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from post-extracted plasma standard (spiked blank plasma extracts after extraction) sample at equivalent concentrations [21]. Recoveries of esculin and esculetin were determined at QC low, QC medium and QC high concentrations, viz., 50, 800, 2500 ng/mL and 2.5, 40.0, 125.0 ng/mL, respectively, whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

2.7. Sample preparation

The solid-phase extraction (SPE) cartridges (ProElut C_{18} 60 mg/3 mL 50/pkg, Dikma Technology, USA) were washed with 3000 μL of methanol followed by 3000 μL of water. 50 μL of rat plasma sample and 10 μL of the scopoletin working solution (1000 ng/mL) were pipetted into the 1.5-mL Eppendorf tubes, and then followed by 440 μL water. The resultant mixture was vortexed for approximately 30 s and spiked into the cartridge under vacuum, then washed with 1000 μL water. A new 1.5-mL Eppendorf tubes were positioned under the SPE cartridge and the compounds were eluted with 1000 μL of methanol. The eluent was evaporated dryness by a gentle stream of nitrogen at 37 °C. The residues were reconstituted in 100 μL of the mobile phase and 5 μL of the sample solution was injected into the LC–MS/MS system for assay.

2.8. Validation procedures

A full validation according to the FDA guidelines was performed for the assay in rat plasma [22].

2.8.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for analyte and IS using the proposed extraction procedure and chromatographic–MS conditions. All the plasma samples were pretreated and analyzed under the same procedure as described.

2.8.2. Matrix effect

The effect of rat plasma constituents over the ionization of esculin, esculetin and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 6$) with the response of analytes from the standard solutions evaporated directly and reconstituted in the mobile phase (10 μL of required working stock sample spiked into 50 μL of methanol instead of blank plasma at equivalent concentrations). The matrix effect for esculin and esculetin was determined at QC of low, medium and high concentrations (50, 800, 2500 ng/mL and 2.5, 40.0, 125.0 ng/mL, respectively), whereas the matrix effect over the IS was determined at a single concentration of 100 ng/mL in six replicates. The matrix effect is implied if the ratio is less than 85% or more than 115% [22].

2.8.3. Linearity and lower limit of quantification (LLOQ)

The calibration curve was acquired by plotting the ratio of peak areas of esculin and esculetin to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 25, 50, 100, 200, 400, 800, 1600, 3200 ng/mL (esculin) and 1.25, 2.50, 5.00, 10.00, 20.00, 40.00, 80.00, 160.00 ng/mL (esculetin). The results were fitted to linear regression analysis using $1/x$ as the weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The LLOQ of the assay was assessed as the lowest concentration on the calibration curve that could be quantitatively determined with an acceptable precision less than 20% and accuracy within ±20%, which was established based on five replicates independent of the QC samples.

2.8.4. Precision and accuracy

Precision and accuracy of the method were evaluated at concentrations of 50, 800, 2500 ng/mL (esculin) and 2.5, 40.0, 125.0 ng/mL (esculetin) plasma. For the evaluation of intra-day precision and accuracy, five aliquots of each sample were analyzed on the same day. For inter-day precision and accuracy, five aliquots of each sample were analyzed on three consecutive days. The criteria for acceptability of the data included accuracy within ±15% standard deviation (SD) from the nominal values and a precision within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision.

2.8.5. Stability

All stability studies were conducted at three concentration levels, i.e. QC low, middle and high, using six replicates at each concentration level. Replicate injections of processed samples were analyzed up to 24 h to establish auto-sampler stability of analyte and IS at 10 °C. The peak areas of analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of esculin and esculetin in the bio-matrix during 8 h exposure at room temperature in rat plasma (bench top) was determined at ambient temperature (25 ± 2 °C). Freeze/thaw stability was evaluated up to three cycles. Freezer stability of esculin and esculetin in rat plasma was assessed by analyzing the QC samples stored at −70 °C for at least 30 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy and precision (85–115%).

2.9. Application to a pharmacokinetic study in rats

Sprague-Dawley rats were purchased from Guangdong Experimental Animal Center (Guangzhou, China) and maintained on a 12 h light-dark cycle with free access to food and water for seven days. The rats were fasted for 12 h and had free access to water before dosing. On the day before the pharmacokinetic study, a polyethylene tube (i.d. 0.58 mm, o.d. 0.965 mm, Becton Dickinson, Sparks, MD, USA) was implanted into the right jugular vein through surgery for collecting the blood sampling. In vivo oral pharmacokinetic study was performed in male Sprague-Dawley rats ($n = 8$, weight range 250–270 g) to demonstrate the applicability of newly developed and validated bio-analytical method. After oral administration of 100 mg/kg esculin in 0.50% sodium carboxy methyl cellulose (CMC-Na) suspension to rats by gavage, serial blood samples (150 μL) were collected before dosing and at 0.05, 0.117, 0.167, 0.250, 0.500, 0.750, 1, 2, 4, 6, 8 and 12 h after oral dosing, and then centrifuged at 3000 × g for 10 min at 20 °C immediately and stored frozen at −70 °C until analysis. Rat plasma (50 μL) samples were spiked with IS and processed as described above. The plasma concentrations of esculin and esculetin at different time points were expressed as mean ± SD. The study was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

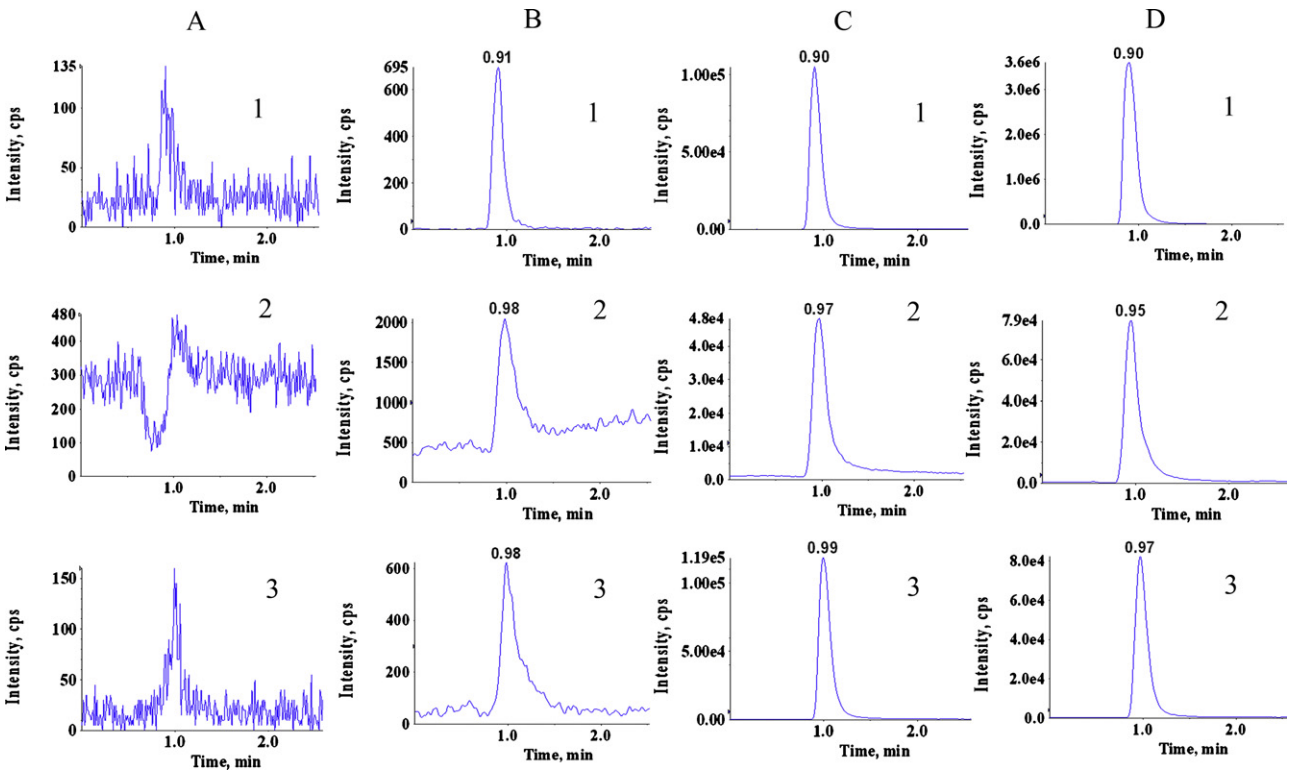

Fig. 2. Representative MRM chromatograms for (A) blank plasma; (B) LLOQ, 0.25 ng/mL for esculin, 1.25 ng/mL for aesculetin and 0.50 ng/mL of esculin, 33.3 ng/mL of aesculetin and 100 ng/mL of IS; (D) real sample 15 min after oral administration of 100 mg/kg esculin. (1) Esculin, (2) aesculetin, (3) scopoletin (IS).

Pharmacokinetic parameters from rat plasma samples were calculated by a non-compartmental statistical model using ONMEM Program version 1.1 (GloboMax Inc., Ellicott City, MD). Blood samples were taken for a period of three to five times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration–time curve (AUC) ratio higher than 80% according to the US Food and Drug Administration (FDA) guidelines. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly obtained from the experimental data. The elimination half-life ($t_{1/2\beta}$) was calculated as $0.693/\beta$ where β is the elimination rate constant calculated from the terminal linear portion of the log plasma concentration–time curve. The area under the plasma concentration–time curve (AUC) from time zero to the last quantifiable time point ($AUC_{0\rightarrow t}$) and from time zero to infinity ($AUC_{0\rightarrow\infty}$) were estimated using the log-linear trapezoidal rule.

3. Results and discussion

3.1. Liquid chromatography

It was necessary in the method development to optimize parameters such as choice of types of reversed-phase chromatographic column and organic solvent, pH, composition of the mobile phase, flow rate. Reversed-phase chromatographic column including Gemini C_{18} (50 mm × 2.0 mm, 5 μm, Phenomenex, USA), Alltima C_{18} (150 mm × 2.1 mm, 5.0 μm, Alltech, USA), Zorbax SB-C_{18} (250 mm × 4.6 mm, 5 μm particle; Agilent, USA) were tested to improve peak shape, increase analyte signal response, and shorten run time. A column with a narrow internal diameter Gemini C_{18} (50 mm × 2.0 mm, 5 μm) was finally selected for the chromatographic separation because under the current LC conditions, the column provided excellent results in terms of response, retention time and peak shapes. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate, acetic acid and formic acid with variable pH range of 3.5–5.5, along with altered flow rates (in the range of 0.15–0.5 mL/min) were tested for complete chromatographic resolution of esculin, esculetin and IS (data not shown). It was also found that the optimal mobile phase consisted of methanol and water containing 0.01% formic acid (80:20, v/v, pH = 3.7) at a flow rate of 0.30 mL/min. Methanol, rather than acetonitrile, was chosen as the organic modifier because it led to lower background noise and resulted in the best resolution. The addition of 0.1% formic acid decreased overall sensitivity and peak area for analyte and IS. The chromatographic run time for the extracted plasma samples was 2.5 min. The retention time for esculin, esculetin and IS were 0.90, 0.95 and 0.97 min, respectively (Fig. 2). Fig. 2 shows typical representative chromatograms from blank plasma, LLOQ of esculin, esculetin and IS (0.25 ng/mL, 1.25 ng/mL and 0.50 ng/mL, respectively), blank plasma spiked with 33.3 ng/mL of esculin, 33.3 ng/mL of esculetin and 100 ng/mL of IS, and from rat plasma obtained 15 min following a 100 mg/kg oral dose of esculin. The chromatogram shows baseline separation of analyte and IS without any interference from endogenous plasma components.

3.2. Mass spectrometry

In order to optimize ESI conditions for esculin, esculetin and IS, quadrupole full scans were carried out in negative ion detection mode. And multiple reactions monitoring (MRM), in which precursor ions are fragmented and unique product ions are measured, enabled selective detection of all compounds simultaneously, in spite of the fact that some ion-pairs were not well resolved chromatographically. During a direct infusion experiment, the mass

Table 2
Marrix effect esculin and esculetin in rat plasma ($n = 6$).

Analyte	Analyte concentration (ng/mL)	Matrix effect (mean ± SD)		
		MC (ng/mL)	RSD (%)	Accuracy (%)
Esculin	50	50.98 ± 2.81	5.50	101.95 ± 8.71
	800	802.80 ± 22.11	2.75	100.35 ± 5.78
	2500	2495.03 ± 115.26	4.62	99.80 ± 5.23
Esculetin	2.5	2.59 ± 0.25	8.05	103.45 ± 8.53
	40.0	41.71 ± 7.73	7.74	104.28 ± 5.50
	125.0	126.13 ± 10.34	5.41	100.91 ± 4.79

MC, measured concentration.

Table 3
Recovery of esculin and esculetin in rat plasma by Dikma C18-SPE method ($n = 6$).

Analyte	Analyte concentration (ng/mL)	Extraction recovery (mean ± SD)		
		MC (ng/mL)	RSD (%)	Recovery (%)
Esculin	50	38.21 ± 3.10	8.10	76.43 ± 8.92
	800	572.55 ± 41.92	7.32	71.57 ± 8.01
	2500	1878.55 ± 120.16	6.40	75.14 ± 7.21
Esculetin	2.5	1.17 ± 0.11	9.71	46.97 ± 8.10
	40.0	18.99 ± 1.09	5.76	47.48 ± 6.44
	125.0	74.35 ± 4.89	6.58	59.48 ± 6.89

MC, measured concentration.

spectra for esculin, esculetin and IS revealed peaks at *m/z* 339.1, 176.9 and 191.0, respectively as ionized molecular ions $[M-H]^-$ (Fig. 1). The daughter spectra of the parent ions showed that the predominant daughter fragments were *m/z* 176.7 for esculin, *m/z* 133.0 for esculetin and *m/z* 175.9 for IS (Fig. 1). Following detailed optimization of mass spectrometry conditions (provided in instrumentation and chromatographic conditions section); the *m/z* 339.1 precursor ion to the *m/z* 176.7 was used for quantification for esculin. Similarly, for esculetin *m/z* 176.9 precursor ion to the *m/z* 133.0 was used for quantification purpose.

3.3. Validation procedures

3.3.1. Matrix effect and extraction recovery

In this study, the matrix effect of esculin and esculetin was evaluated by analyzing QC low (50 ng/mL, 2.5 ng/mL), QC medium (800 ng/mL, 40 ng/mL) and QC high (2500 ng/mL, 125 ng/mL) samples. The results are summarized in Table 2. No significant matrix effect for esculin, esculetin and IS were observed indicating that no co-eluting substance influenced the ionization of the analytes.

Protein precipitation followed by liquid/liquid extraction has been commonly applied in biological fluids [22]. Different solvents have been used for the sample extraction and most of them are based on either acetonitrile or methanol. Esculin and esculetin belong to coumarin. Esculin is hydrosoluble and soluble in hot water, methanol and ethanol. Esculetin is soluble in ethanol, dilute alkali solution, slightly soluble in n-butyl alcohol and ethyl acetate, insoluble in diethyl ether and chloroform. Therefore, we tested the extraction recoveries of esculin and esculetin using different organic solvents including acetonitrile, methanol, ethyl acetate, n-butyl alcohol and their mixtures. Results showed that the extraction recovery was below to the LLOQ when using protein precipitation by acetonitrile and methanol or liquid–liquid extraction with ethyl acetate and n-butyl alcohol in the plasma concentration of esculetin below 40 ng/mL (Supplementary material, Table 3-1). Under our LC-conditions we have observed a bad peak shape after pretreating by liquid–liquid extraction or protein precipitation. Consideration of their hydrophilic characterization, different kinds of solid phase extraction columns including C_2, C_8, cyan (CN), Silica, ProElut anion exchange inverse column (PXA) and ProElut hydrophilic–lipophilic balance column (PLC) were selected to investigate the recovery in the present study. Interestingly, C_{18}-SPE method has the higher extraction recovery than that of other SPE methods. Subsequently, we further tested C_{18}-SPE columns of different brands containing Dikma, Phenomenex and Agilent. The results show the higher recoveries of esculin and esculetin were found when used the Dikma C_{18}-SPE column. Table 3 shows the mean extraction recoveries are 76.43 ± 8.92%, 71.57 ± 8.01% and 75.14 ± 7.21% for esculin at the concentrations of 50, 800, 2500 ng/mL and 46.97 ± 8.10%, 47.48 ± 6.44% and 59.48 ± 6.89 for esculetin at the concentrations of 2.5, 40, 125 ng/mL, respectively. The absolute recovery of IS at 100 ng/mL was 74.92%.

3.3.2. Linearity and LLOQ

The calibration curves were obtained by plotting the peak area ratio of the analytes to IS against the corresponding concentration of the analytes in the freshly prepared plasma calibrators. The plasma calibration curves of esculin and esculetin were constructed using eight calibration standards (viz., 25–3200 ng/mL, 1.25–160 ng/mL, respectively). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/x$). The average regression ($n = 5$) of esculin and esculetin was found to be ≥0.9994 and ≥0.9996, respectively.

LLOQ samples of six different rats' plasma, independent from the calibration curve were analyzed and found to be 0.5 ng/mL and 1.25 ng/mL, with an accuracy of 105.1%, 106.2% and within- and between-run precisions of 5.4%, 6.7% and 6.3%, 7.4%, respectively. The LLOQ was sufficient for pharmacokinetic studies after oral administration of esculin to rats.

3.3.3. Accuracy and precision

The intra-day precision and accuracy was determined by the replicate analyses of QC samples ($n = 5$) at three level concentrations during the three separate days. One replicate of the QC samples at each concentration level from three separate validation

Table 4
Intra-day and inter-day precision and accuracy for assay of esculin and esculetin in plasma.

Analyte	Analyte concentration (ng/mL)	Intra-day ($n = 5$) (mean ± SD)			Inter-day ($n = 3$) (mean ± SD)		
		MC[a] (ng/mL)	RSD (%)	A[b] (%)	MC[a] (ng/mL)	RSD (%)	A[b] (%)
Esculin	50	53.53 ± 3.89	7.27	107.05 ± 9.08	52.46 ± 4.48	8.53	104.91 ± 8.97
	800	810.04 ± 26.76	3.30	101.26 ± 7.24	804.74 ± 34.48	4.28	100.59 ± 7.86
	2500	2586.30 ± 80.55	3.11	103.45 ± 5.33	2565.45 ± 97.13	3.79	102.62 ± 5.59
Esculetin	2.5	2.51 ± 0.20	8.05	100.46 ± 7.98	2.50 ± 0.22	8.73	99.97 ± 6.48
	40.0	39.81 ± 1.41	3.55	99.52 ± 7.11	39.50 ± 3.15	7.96	98.74 ± 7.06
	125.0	126.95 ± 6.51	5.12	101.56 ± 4.62	125.48 ± 6.51	5.18	100.38 ± 4.56

[a] MC, measured concentration.
[b] A, accuracy.

Table 5
Stability of esculin and esculetin in plasma ($n = 5$).

Analyte	Analyte concentration (ng/mL)	Three freeze–thaw cycles (mean ± SD)			Short-term stability for 8 h in plasma (mean ± SD)			Auto-sampler stability for 24 h (mean ± SD)			long-term stability for 30 days at −70 °C (mean ± SD)		
		MC[a] (ng/mL)	RSD (%)	A[b] (%)	MC[a] (ng/mL)	RSD (%)	A[b] (%)	MC[a] (ng/mL)	RSD (%)	A[b] (%)	MC[a] (ng/mL)	RSD (%)	A[b] (%)
Esculin	50	52.56 ± 4.53	5.61	105.12 ± 6.17	52.89 ± 4.81	6.10	105.78 ± 6.01	50.21 ± 1.88	3.74	100.43 ± 2.97	51.72 ± 4.01	7.76	103.44 ± 5.15
	800	807.54 ± 17.18	2.13	100.94 ± 4.85	800.10 ± 18.62	2.33	100.01 ± 4.68	800.12 ± 20.36	2.55	100.02 ± 2.42	803.88 ± 22.12	2.75	100.49 ± 6.01
	2500	2528.92 ± 55.23	2.18	101.16 ± 3.48	2505.00 ± 112.11	2.48	100.20 ± 2.97	2500.09 ± 49.36	1.97	100.00 ± 1.67	2549.60 ± 199.72	3.83	101.98 ± 4.88
Esculetin	2.5	2.55 ± 0.22	8.54	101.93 ± 5.35	2.48 ± 0.11	4.35	99.26 ± 7.49	2.56 ± 0.10	4.01	102.59 ± 3.01	2.63 ± 0.16	6.14	105.14 ± 9.02
	40.0	39.15 ± 1.95	4.98	97.87 ± 6.01	42.68 ± 3.93	5.22	106.70 ± 4.95	40.55 ± 1.28	3.15	101.37 ± 2.61	41.88 ± 2.27	5.42	104.70 ± 5.74
	125.0	132.01 ± 5.05	3.83	105.61 ± 3.15	126.78 ± 6.31	4.98	101.42 ± 2.65	125.47 ± 1.94	1.55	100.38 ± 2.02	127.29 ± 6.48	5.09	101.83 ± 4.89

[a] MC, measured concentration.
[b] A, accuracy.

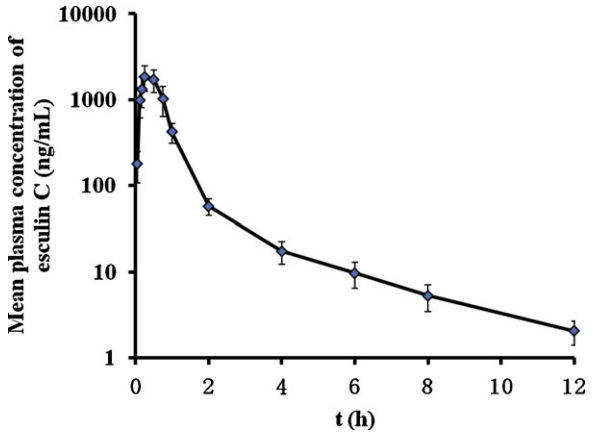

Fig. 3. Plasma concentration–time profiles of esculin in rat plasma after oral administration dose of 100 mg/kg of esculin to rats ($n = 8$).

batches was used to evaluate the inter-day precision. The assay precision and accuracy results are shown in Table 4. The intra-day precision was within 8.05% and the inter-day precision was within 8.73%. The assay accuracy was 98.74–107.05% of the nominal values.

3.3.4. Stability

The stability of esculin and esculetin in plasma was investigated. The stability experiments were aimed at testing the effects of possible conditions that the samples might experience between collection and analysis. Stability results are summarized in Table 5. Esculin and esculetin in rat plasma were found to be stable after being placed at room temperature for 8 h. Testing of autosampler stability of extracting samples indicated that esculin and esculetin were stable when kept in the autosampler (10 °C) for 24 h. Three cycles of freeze and thaw for QC samples indicated that esculin and esculetin were stable in plasma. QC samples were stable when stored frozen at −70 °C for at least 30 days.

3.4. Pharmacokinetic study

We applied the newly developed LC–ESI-MS/MS method to the pharmacokinetic study of esculin and esculetin and successfully obtained a series of the pharmacokinetic data of esculin and esculetin in eight Sprague-Dawley rats after oral administration of 100 mg/kg.

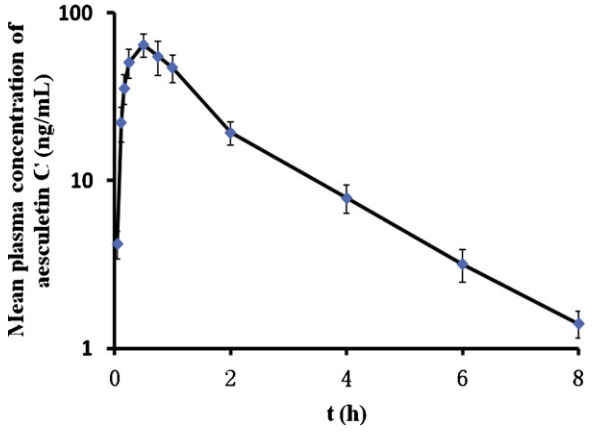

Fig. 4. Plasma concentration–time profiles of esculetin in rat plasma after oral administration dose of 100 mg/kg of esculin to rats ($n = 8$).

Table 6
Main pharmacokinetic parameters of esculin and esculetin after oral administration of 10 mg/kg of esculin to rats ($n = 8$).

Analyte	Parameters (mean ± SD)					
	β (h^{-1})	$t_{(1/2\beta)}$ (h)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0\to12}$ (ng/mL h)	$AUC_{0\to\infty}$ (ng/mL h)
Esculin	0.27 ± 0.02	2.60 ± 0.23	0.25 ± 0.03	1850.39 ± 129.71	1571.33 ± 128.06	1579.01 ± 116.12
Esculetin	0.44 ± 0.04	1.58 ± 0.12	0.50 ± 0.05	64.62 ± 5.13	124.24 ± 9.71	127.44 ± 11.64

Mean plasma concentration–time profile of esculin and esculetin after oral administration of 100 mg/kg of esculin to rats was shown in Figs. 3 and 4, and the major pharmacokinetic parameters of esculin and esculetin after oral administration were calculated by a non-compartmental model and are presented in Table 6.

4. Conclusion

This is the first report on the pharmacokinetic study of esculin and esculetin (the aglycone metabolites of esculin) with a total running time of 2.5 min per sample and LLOQ of 0.25 ng/mL and 1.25 ng/mL, respectively. Therefore, a simple, sensitive, specific, accurate and reproducible LC–MS/MS method used to perform pharmacokinetic studies in rat plasma using structurally close IS was developed and validated in the quantification of CF.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.08.027.

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