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Short communication

Simultaneous determination of pimpinellin, isopimpinellin and phellopterin in rat plasma by a validated UPLC–MS/MS and its application to a pharmacokinetic study after administration of *Toddalia asiatica* extract

Zhigang Liu [a,b], Minyan Jiang [a], Xiumei Lu [a], Feng Qin [a], Yang Song [a], Jing Wen [a], Famei Li [a,*]

[a] Department of Analytical Chemistry, Shenyang Pharmaceutical University, Shenyang 110016, China
[b] Department of Biological and Environmental Engineering, Guiyang University, Guiyang 550005, China

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ABSTRACT

A rapid and selective ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was developed for simultaneous determination of three bioactive coumarins of *Toddalia asiatica* extract including pimpinellin, isopimpinellin and phellopterin in rat plasma for the first time. Phenacetin was used as the internal standard (IS). Plasma samples were extracted by liquid–liquid extraction with methyl tert-butyl ether. The chromatographic separation was carried out on an ACQUITY UPLC^{TM} BEH C_{18} column with an isocratic mobile phase consisting of methanol-5 mmol/L ammonium acetate (65:35, v/v). The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) via electrospray ionization (ESI) source with positive ionization mode. The method was linear for all analytes over investigated range with all correlation coefficients greater than 0.9942. The lower limits of quantification (LLOQ) were 25.0 ng/mL for pimpinellin, 10.0 ng/mL for isopimpinellin and 5.00 ng/mL for phellopterin. The intra- and inter-day precision (RSD%) was within 12% and the accuracy (RE%) ranged from −2.3% to 5.5%. The rapid and sensitive method was fully validated and successfully applied to the pharmacokinetic study of pimpinellin, isopimpinellin and phellopterin in rats following oral administration of *Toddalia asiatica* extract.

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1. Introduction

Toddalia asiatica (L.) Lam. (Rutaceae) is widely used as a folk medicine in many Asian and Africa countries. The root and its bark have been used for the treatment of traumatic injury, rheumatism, gall etc. [1,2]. In Miao tribes of southern China, it was extensively used to treat fever, malaria, cholera, diarrhoea and indigestion [3]. It was reported that *Toddalia asiatica* extract significantly inhibited influenza type A virus [4], as well as displayed outstanding antibacterial and antifungal activity [5]. Phytochemical investigation has found that coumarins are important active constituents most contributing to the pharmacological efficacy of *Toddalia asiatica* [6–9]. Pimpinellin, isopimpinellin and phellopterin are three major coumarins present in *Toddalia asiatica* extract which demonstrated activities like anticancer, vasodilative and antioxidant etc. [10–15]. Therefore, it is essential to develop a quantitative method for determining these coumarins in plasma samples for the purpose of studying the mechanism of action.

Several analytical methods for coumarins including one or two of pimpinellin, isopimpinellin and phellopterin have been reported [16–19]. Zhang et al. [17] published an LC–MS method to determine 17 major components including pimpinellin in Yuanhu Zhitong tablet. Wang et al. [18] developed an HPLC–UV method for analysis 9 coumarins including isopimpinellin in human urine. However, there are few papers where the three components have been determined in rat plasma, and very little attention has been devoted to the pharmacokinetic studies of these bioactive components. Only one report related to the quantitative determining of isopimpinellin in rat plasma was found in the literature [16], pimpinellin was studied as internal standard, this method is lack of full validation.

The present paper reports, for the first time, the development and validation of a rapid and selective UPLC–MS/MS method for the simultaneous determination of pimpinellin, isopimpinellin and phellopterin in rat plasma with good accuracy. The total run time was 4.5 min per sample. The method was fully validated and applied to the pharmacokinetic study after oral administration of *Toddalia asiatica* extract to rats. It was expected that the results of this study would provide some references to the apprehension of the action mechanism and further pharmacological study of *Toddalia asiatica*.

* Corresponding author. Tel.: +86 24 2398 6289; fax: +86 24 2398 6289.
E-mail address: lifamei@syphu.edu.cn (F. Li).

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2. Experimental

2.1. Chemicals and materials

The root bark of *Toddalia asiatica* was collected in a valley, located in Huaxi district, Guizhou province, China. The raw material was identified by Professor Pengmin Gao (Guiyang University, Guiyang, China) according to the Chinese Materia Medica (vol. Miao medicine) [3] and dried in the sunlight according to the Pharmacopoeia of the People's Republic of China. Reference standard of pimpinellin, isopimpinellin and phellopterin were isolated and purified in our laboratory. On the basis of UV, NMR and MS analysis, the structures of isolated reference standards were confirmed, and their purities determined using HPLC–PDA were over 98.0% by comparing with the reference standard (obtained from Shanghai Taoto Biotech Co., China). Phenacetin (internal standard, IS, 99.5% of purity) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Methanol of HPLC grade was obtained from Tedia (Fairfield, OH, USA). Ammonium acetate (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The chromatography was performed on an ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with cooling auto-sampler and column oven enabling temperature control. An ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed and the column temperature was maintained at 40 °C. The mobile phase was composed of methanol-5 mmol/L ammonium acetate (65:35, v/v) with a flow rate set at 0.20 mL/min. The auto-sampler was conditioned at 4 °C and the injection volume was 10 μL.

2.2.2. Mass spectrometry

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro^{TM} API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode. The quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 247.1 → 231.1 for pimpinellin, m/z 247.1 → 217.0 for isopimpinellin, m/z 301.0 → 233.1 for phellopterin and m/z 180.0 → 110.0 for phenacetin (I.S.), respectively, with a scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 1.5 kV, cone voltage 40 V, source temperature 110 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 450 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.81×10^{-3} mbar. The optimized collision energy for pimpinellin, isopimpinellin, phellopterin and phenacetin was 23, 23, 12 and 20 eV, respectively. All data collected in centroid mode were acquired and processed using MassLynx^{TM} NT 4.1 software with QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

2.3. Preparation of Toddalia asiatica extract

The dried powder of *Toddalia asiatica* (100 g) was extracted twice under reflux with 75% ethanol (1:8, w/v) for 1 h per time. The extraction solutions were combined and filtered, and then the ethanol was removed under reduced pressure. The residuary solution was then diluted with water to get the *Toddalia asiatica* extract with a concentration equivalent to 1.0 g/mL of the raw *Toddalia asiatica* material.

The contents of the three coumarins in *Toddalia asiatica* extract solution were measured quantitatively by external standard method using the same chromatography conditions as described above. The contents of pimpinellin, isopimpinellin and phellopterin in the extract were 10.1, 3.9 and 5.0 mg/mL, respectively.

2.4. Preparation of calibration standard and quality control samples

A mixed stock solution containing 500 μg/mL of pimpinellin, 200 μg/mL of isopimpinellin and 100 μg/mL of phellopterin was prepared by dissolving required amount of the chemical reference substance in methanol. The stock solution was serially diluted with a mixture of methanol–water (65:35, v/v) to provide working standard solutions of desired concentrations. The internal standard solution was prepared by diluting the I.S. stock solution with a mixture of methanol–water (65:35, v/v) to 1.10 μg/mL. All the solutions were stored at 4 °C.

Calibration standards were prepared daily by evaporating 50 μL of the appropriate standard working solutions to dryness and then fully mixing with 100 μL of blank plasma giving concentrations of 25.0, 50.0, 100, 250, 500, 1000, and 2500 ng/mL for pimpinellin; 10.0, 20.0, 40.0, 100, 200, 400, and 1000 ng/mL for isopimpinellin and 5.00, 10.0, 20.0, 50.0, 100, 200, and 500 ng/mL for phellopterin, respectively. Quality control (QC) samples were independently prepared in the same way at 62.5, 375, 2000 ng/mL for pimpinellin, 25.0, 150, 800 ng/mL for isopimpinellin and 12.5, 75.0, 400 ng/mL for phellopterin. The standards and quality controls were extracted on each analysis day with the same procedures for plasma samples as described below.

2.5. Plasma sample preparation

Fifty microliters of IS solution were pipetted into 10 mL clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was vertex-mixed with 100 μL of plasma for 30 s and 3 mL of methyl tert-butyl ether was added. The mixture was vortex-mixed for 60 s. After centrifugation at 3500 × g for 10 min, the upper organic layer was then transferred into a clean glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of methanol–water (65:35, v/v), and transferred to an autosampler vial and an aliquot of 10 μL was injected onto the UPLC–MS/MS system for analysis.

2.6. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery and stability according to FDA guidance for validation of bioanalytical methods [22].

The selectivity of the method was evaluated by comparing the chromatograms of six different batches of blank plasma. Blank samples were extracted and analyzed by UPLC–MS/MS for potential interfering peaks within the range of the retention time of each analyte.

Calibration curves were prepared by assaying standard plasma samples at seven concentration levels, duplicated samples for each level were prepared and assayed on three separate days. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to I.S. versus the nominal concentration (x) of analytes with weighted ($1/x^2$) least square linear regression.

The lower limit of quantification is defined as the lowest concentration on the calibration curve with an acceptable accuracy (relative error, R.E.) within ±20% and a precision (relative standard deviation, R.S.D.) below 20%.

The accuracy and precision were assessed by analyzing QC samples in six replicates at three concentrations levels on three

consecutive days. The precision was expressed as R.S.D. and the accuracy as R.E.

The extraction efficiency of the three analytes was determined by analyzing six replicates of QC samples at three concentration levels. The extraction recovery was calculated by comparing the peak areas obtained from extracted spiked samples with those of samples spiked post-extraction at corresponding concentrations. The matrix effect was evaluated by comparing the peak areas of the analytes obtained from six plasma samples with the analytes spiked after extraction, at three concentration levels, to those from the neat standard solutions at the same concentrations. The extraction recovery and matrix effect of I.S. were also evaluated using the same procedure.

The stability of analytes in rat plasma was assessed by analyzing three replicates of low, mid and high QC samples during the sample storage and processing procedures. The freeze–thaw stability examination was performed by subjecting the unextracted QC samples to three freeze (−20 °C)–thaw (room temperature) cycles. The QC samples were stored at ambient temperature for 4 h to determine short-term stability. The post-preparation stability was studied by analyzing the extracted QC samples kept in the autosampler at 4 °C for 12 h. All stability testing QC samples were determined by using a calibration curve of freshly prepared standards. The determined concentrations were compared with the nominal values.

2.7. Pharmacokinetic study

This validated method was applied to monitor the plasma concentrations of pimpinellin, isopimpinellin and phellopterin in rats after single oral administration of *Toddalia asiatica* extract at a dose of 0.5 g/kg body weight. Six male Wistar rats, weighing 200 ± 20 g, were supplied by Shenyang Pharmaceutical University. The experimental protocols were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University. The rats were housed under controlled environmental conditions (temperature: 25 ± 2 °C, relative humidity: 50 ± 10%) with free access to food and water until 12 h prior to experiments. Blood samples (0.25 mL) were collected from the suborbital vein into heparinized tubes at 0, 0.17, 0.33, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h after oral administration of *Toddalia asiatica* extract. All blood samples were immediately centrifuged for 10 min at 12,000 rpm, and the plasma was transferred into clean tubes and stored at −20 °C until analysis. Plasma samples were spiked with IS, and processed as described in Section 2.5 above. Along with the plasma samples, QC samples were distributed among calibrators and unknown samples in the analytical run.

The maximum plasma concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were noted directly from the measured data. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of mass spectrometry

A standard solution of the analytes and I.S. were directly infused along with the mobile phase into the mass spectrometer with electrospray ionization (ESI) as the ionization source. The response observed in positive ionization mode was higher than that in negative ionization mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ m/z 247.1, 247.1, 301.1 and 180.0 for pimpinellin, isopimpinellin, phellopterin and I.S., respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity of protonated molecule of analytes. Multiple reaction monitoring (MRM) using the precursor → product ion transitions of m/z 247.1 → m/z 231.1, m/z 247.1 → m/z 217.0, m/z 301.0 → m/z 233.1 and m/z 180.0 → m/z 110.0 were employed for quantification of pimpinellin, isopimpinellin, phellopterin and I.S., respectively (Fig. 1).

3.1.2. Optimization of chromatography

Chromatographic conditions were optimized to obtain high sensitivity and sample throughput. The mobile phase systems of acetonitrile–water and methanol–water in various proportions were tested. The responses of analytes were higher with methanol–water as the mobile phase than those with acetonitrile–water. Methanol proportion in the mobile phase from 60% to 80% was considered in the followed experiment. In view of the response, retention times and peak shapes of both analytes and I.S., 65% methanol was the best.

Electrolyte modification of mobile phase can significantly improve the ESI efficiency resulting in enhanced analyte response. Both analytes and I.S. were found to have the highest response and the best peak shapes in the mobile phase containing 5 mmol/L ammonium acetate. The very sharp chromatographic peaks indicated a high chromatographic efficiency of the UPLC system, which pushed the limits of both analysis speed and peak capacity without compromising resolution. Both analytes and I.S. were rapidly eluted with retention time of 2.1, 1.7, 3.6 and 1.4 min for pimpinellin, isopimpinellin, phellopterin and phenacetin (I.S.), respectively, and a total run time of 4.5 min per sample, which was short than that in the literature [16] and it greatly met the requirement for high sample throughput in bioanalysis.

3.1.3. Optimization of extraction conditions

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. Protein precipitation (PPT) is often used for the preparation of biological samples with the advantages of simplicity and time saving [16], however, PPT may cause high noise level and interference by endogenous substances [20]. LLE can produce purified as well as concentrated samples and improve the sensitivity and robustness of the assay [21]. In the course of our method development, protein precipitation was initially developed with methanol or acetonitrile, which could not completely eliminate the interferences from the sample matrix. Consequently, liquid–liquid extraction was employed as it offered purer sample and low matrix effect. Several extraction solvents such as diethyl ether, diethyl ether–dichloromethane and methyl tert-butyl ether were investigated. Because of the highest extraction efficiency and lowest noise level, methyl tert-butyl ether was employed as the extraction solvent.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method towards endogenous plasma matrix was evaluated with plasma of six rats. Representative chromatograms obtained from blank plasma, blank plasma spiked with the analytes (at LLOQs) and I.S., and plasma sample after oral administration of *Toddalia asiatica* extract are shown in Fig. 2. Due to the efficient sample treatment and high selectivity of MRM, no obvious interferences from endogenous plasma substances were observed under the described chromatographic conditions.

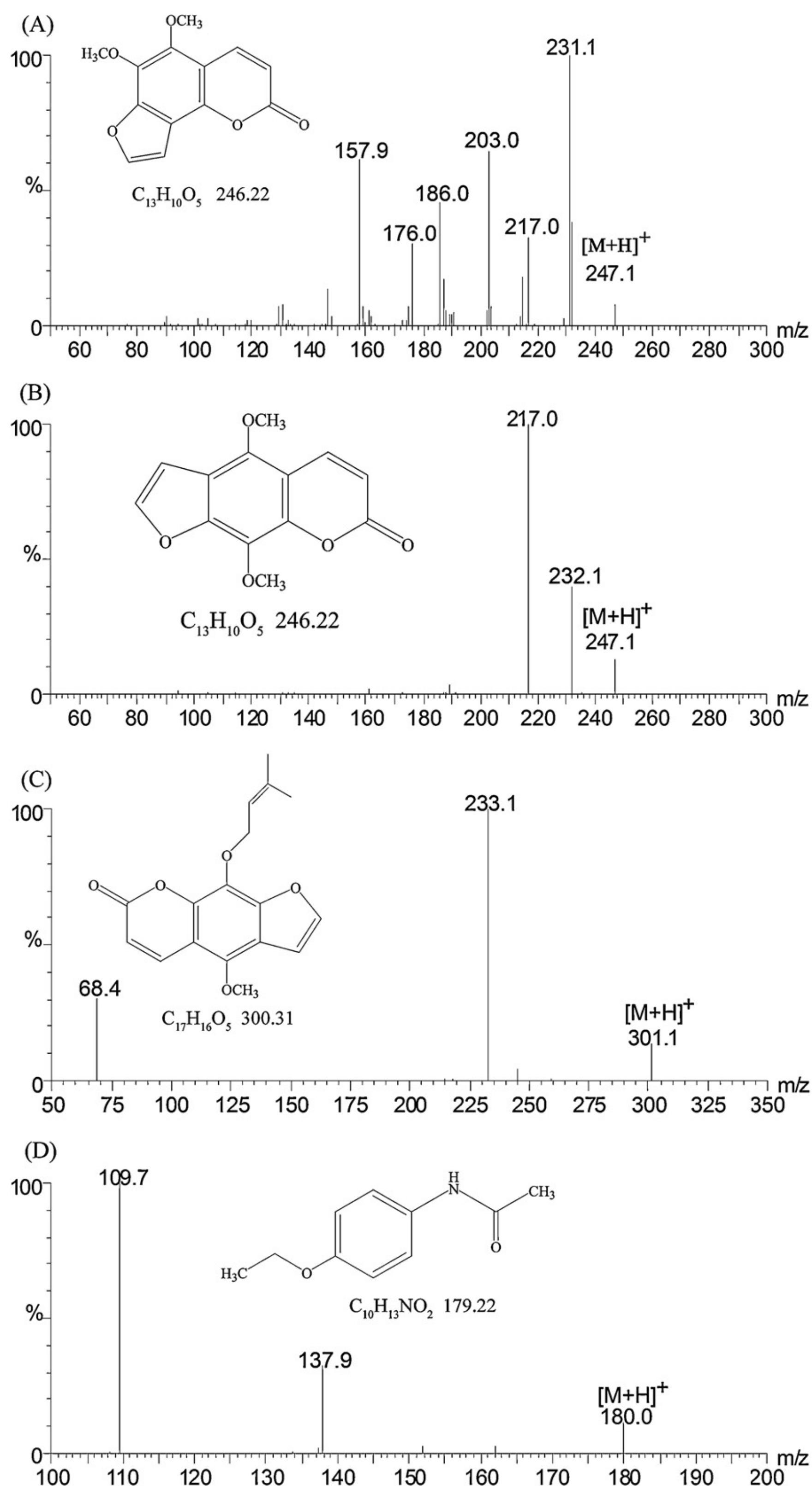

Fig. 1. The product ion scan spectra and chemical structures of pimpiinellin (A), isopimpinellin (B), phellopterin (C) and phenacetin (D; IS).

3.2.2. Linearity and calibration curve

The regression equation, correlation coefficients and linearity ranges for the three analytes are shown in Table 1. The results showed that there was excellent correlation between the ratio of peak area and concentration for each compound within the test ranges. The LLOQs for pimpinellin, isopimpinellin and phellopterin were 25.0 ng/mL, 10.0 ng/mL and 5.00 ng/mL, indicating that this method is sensitive for the quantitative evaluation of the three compounds. The residuals (difference between the back-calculated concentration of the calibration standard and its nominal

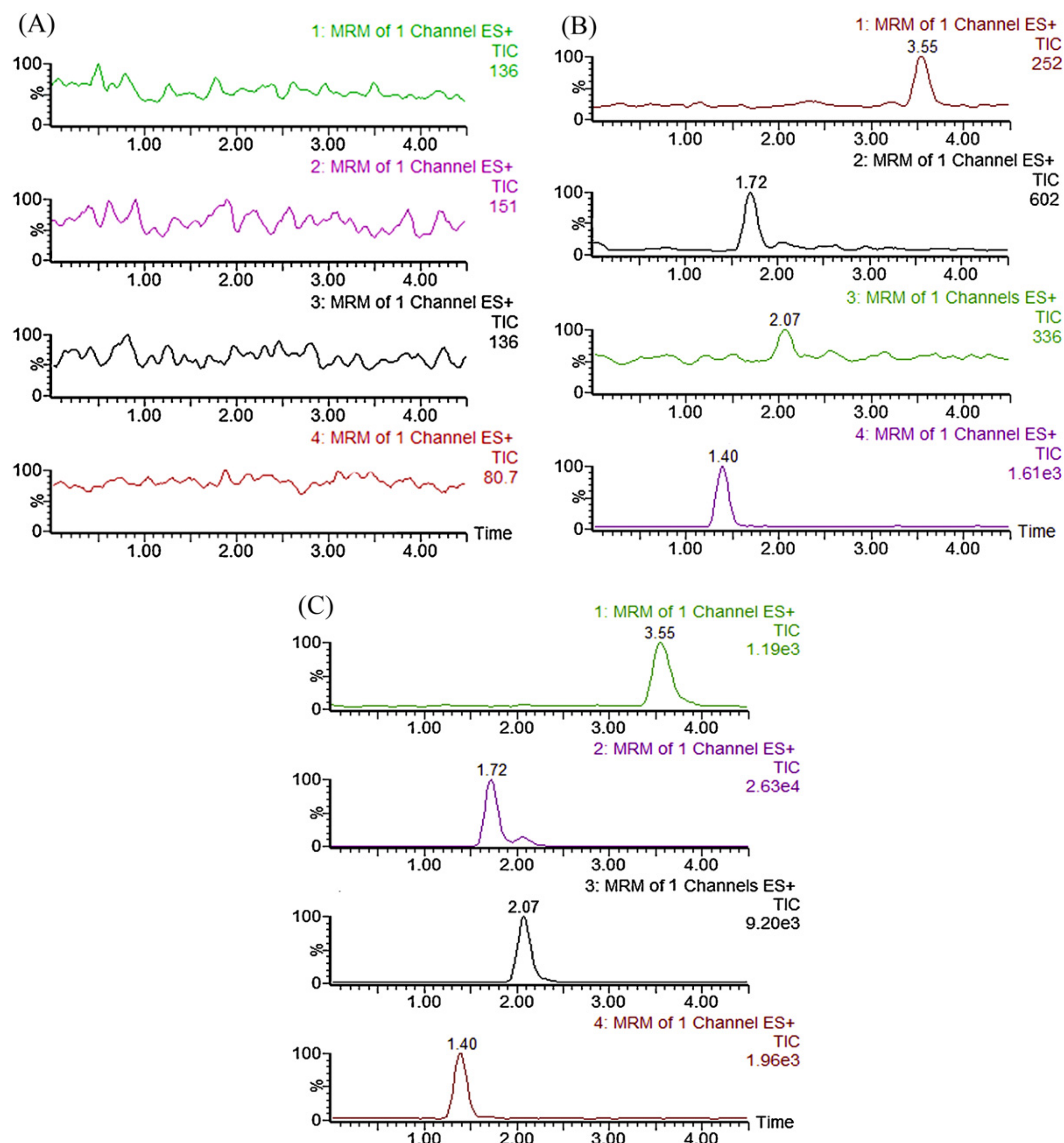

Fig. 2. Representative MRM chromatograms of phellopterin (1), isopimpinellin (2), pimpinellin (3) and phenacetin (4; IS) in rat plasma. (A) blank plasma, (B) blank plasma spiked with the three analytes at LLOQ and IS and (C) plasma sample 1 h after oral administration of *Toddalia asiatica* extract. The retention times of phenacetin, isopimpinellin, pimpinellin and phellopterin were 1.40, 1.72, 2.07 and 3.55 min, respectively.

concentration) were no more than ±15% at all concentrations except at the LLOQ level and no more than 20% at the LLOQ level which demonstrated that the values were all within the acceptable range.

3.2.3. Precision and accuracy

The results of the intra- and inter-day precision and accuracy of the three analytes in LLOQ and QC samples are summarized in Table 2. The intra- and inter-day precisions were within the range from 5.2% to 11% and 3.9% to 12%, respectively. The accuracy derived from QC samples was within ±5.5% for each QC level of the three analytes. The assay values on both intra- and inter-day were all within the acceptable range.

3.2.4. Extraction recovery and matrix effect

The mean recoveries were 75.4 ± 12.7%, 73.8 ± 5.3% and 69.2 ± 2.5% for pimpinellin at three QC levels, 78.3 ± 10.2%, 73.5 ± 3.4% and 76.1 ± 2.8% for isopimpinellin and 82.7 ± 13.8%,

Table 1
The regression equation, linear ranges and LLOQs of pimpinellin, isopimpinellin and phellopterin.

Components	LLOQ (ng/mL)	Linear range (ng/mL)	Linear regression equation	Correlation coefficient (r)
Pimpinellin	25.0	25.0–2500	$Y = 0.1611X - 0.0138$	0.9944
Isopimpinellin	10.0	10.0–1000	$Y = 0.1846X + 0.0135$	0.9950
Phellopterin	5.00	5.00–500	$Y = 0.8817X - 0.0552$	0.9942

Table 2
Precision and accuracy for the determination of pimpinellin, isopimpinellin and phellopterin in rat plasma samples (n = 3 days, replicates per day).

Analyte	Added (ng/mL)	Found (ng/mL)	Intra-dayR.S.D. (%)	Inter-dayR.S.D. (%)	Relative error (%)
Pimpinellin	25.0	25.9	10	11	3.5
	62.5	62.3	7.0	7.9	−2.3
	375	382	8.3	8.6	1.8
	2.00×10^3	2.05×10^3	6.7	14	2.6
Isopimpinellin	5.00	5.10	11	11	3.4
	12.5	13.0	8.7	3.9	1.3
	75.0	73.1	8.1	4.6	−2.6
	400	416	8.7	9.1	4.0
Phellopterin	10.0	10.4	7.5	10	3.6
	25.0	26.0	10	5.6	3.9
	150	158	5.2	4.2	5.5
	800	792	6.8	12	−1.6

75.7 ± 3.9% and 81.2 ± 11.9% for phellopterin, respectively. The mean recovery of I.S. was 78.3 ± 10.2%.

Matrix effect is due to co-elution of some components present in biological samples. Thus, the evaluation of matrix effect from the influence of co-eluting components on analyte ionization is necessary for a UPLC–MS/MS method.

The possibility of a matrix effect caused by ionization competition between the analytes and co-eluents exists when using MS/MS for analysis. To evaluate the matrix effect, the peak areas of blank plasma extracts spiked with analyte post-extraction (A) were compared with those of the standard solutions dried directly and reconstituted with mobile phase (B). All the ratios (A/B × 100)% were between 87.4% and 112.3%, which indicated that the co-eluting matrix components had little effect on the ionization of the analytes and IS.

3.2.5. Stability

Satability of the three analytes during the sample storing and processing procedures was fully evaluated by analysis of QC samples. The results of stability tests indicated that the three analytes were stable in plasma samples for 4 h at room temperature (RE: −9.6% to 12%, RSD < 9.8%) and within three freeze–thaw cycles (RE: −11% to 11%, RSD < 12%). The analytes were stable for 12 h in autosampler after sample preparation (RE: −13% to 7.8%, RSD < 10%).

3.3. Pharmacokinetic study

This validated UPLC–MS/MS method was successfully applied to the pharmacokinetic study of pimpinellin, isopimpinellin and phellopterin in rat plasma following oral administration of *Toddalia asiatica* extract at a dose of 0.5 g/kg. The mean plasma concentration–time curves of pimpinellin, isopimpinellin and phellopterin are presented in Fig. 3. T_{max} and C_{max} were 1.83 ± 0.35 h and 1623 ± 521 ng/mL for pimpinellin, 1.33 ± 0.31 h and 882 ± 234 ng/mL for isopimpinellin, 0.83 ± 0.25 h and 63.9 ± 25.7 ng/mL for phellopterin. $T_{1/2}$ of the three analytes was 1.71 ± 0.51, 1.60 ± 0.50 and 3.28 ± 0.36 h, respectively. AUC_{0-t} and $AUC_{0-\infty}$ were 511 and 521 μg h/mL for pimpinellin, 234 and 237 μg h/mL for isopimpinellin, 15.2 and 16.6 μg h/mL for phellopterin. This is the first report of pharmacokinetic studies of pimpinellin, isopimpinellin and phellopterin in vivo following the oral administration of *Toddalia asiatica* extract.

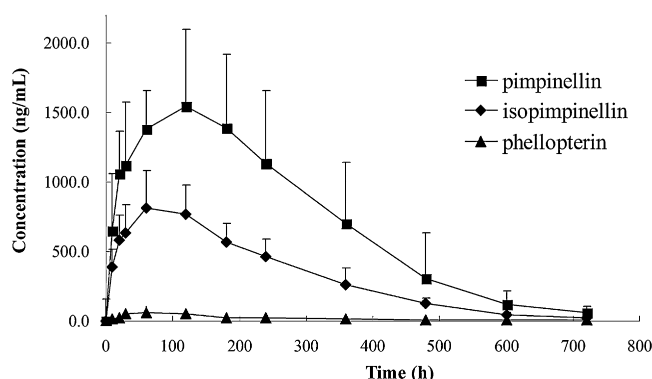

Fig. 3. Mean plasma concentration–time profiles for pimpinellin, isopimpinellin and phellopterin in rats after oral administration of *Toddalia asiatica* extract.

4. Conclusion

In the present paper, a fast and specific UPLC–MS/MS method was developed for simultaneous analysis of pimpinellin, isopimpinellin and phellopterin in rat plasma for the first time. A short analysis time of 4.5 min per sample and a relatively simple sample preparation procedure with one-step liquid–liquid extraction showed greater simplicity and efficiency for analyzing a large number of plasma samples. The method has been successfully applied to the pharmacokinetic study of the three bioactive coumarins in rats following oral dose of *Toddalia asiatica* extract.

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