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ORIGINAL RESEARCH PAPER

Simultaneous quantification of five bioactive 16-deoxybarringtonenol C triterpenoid saponins in rat plasma by HPLC-MS: Application to a pharmacokinetic study after oral administration of *Xanthoceras sorbifolia* Bunge husks extract

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ABSTRACT

In this study, a simple and rapid liquid chromatography-mass spectrometry method was developed to simultaneously determinate five 16-deoxybarringtonenol C triterpenoid saponins with the potential of neuroprotection in rat plasma following the oral administration of the *Xanthoceras sorbifolia* Bunge husks extract. With digoxin as the internal standard, the plasma samples were pre-treated by ethyl acetate-isopropanol (1:1, v/v). The chromatographic separation of the five analytes was performed using a Phenomenex C18 column (250 mm × 4.6 mm, 5.0 mm) with a mobile phase of 0.05% formic acid (A)-acetonitrile (B). The mass spectrometric detection was carried out in the selected ion mode in positive ionization. The extraction recoveries of the five analytes were all over 71.28%. The established method was fully validated in line with the ICH and Food and Drug Administration (FDA) guidelines and successfully applied to the pharmacokinetic study on the five analytes in rat plasma. The terminal half-life ($t_{1/2}$) of the five analytes was 2.92 ± 0.57 , 5.52 ± 1.75 , 2.48 ± 0.62 , 2.95 ± 0.94 , and 2.34 ± 0.81 , respectively. This study was purposed to investigate the oral pharmacokinetic parameters and gain an in-depth insight into the reasonable preclinical use of the husks extract derived from *X. sorbifolia* Bunge.

KEYWORDS

the husks extract of *Xanthoceras sorbifolia* Bunge, 16-deoxybarringtonenol C triterpenoid saponins, HPLC-MS, pharmacokinetics

INTRODUCTION

Xanthoceras sorbifolia Bunge (*X. sorbifolia*), classed into *Sapindaceae*, is known as a woody oil species extensively distributed in the north of China [1]. In folk, its woods are used for rheumatism, and its fruit seeds are applied for pediatric nocturia [1, 2]. In recent decades, its fruit husks have attracted increasing attention due to its potential value in preserving cognitive function. Up to now, both husks extract and Xanthoceraside (a bioactive saponin in the husks) have been widely studied for their effectiveness in neuroprotection in vivo or in vitro experimental models [3–13]. Thus, it is of practical significance for the husks to be further developed and applied for making full use of their potential medicinal value in improving cognitive function.

At present, a variety of chemicals have been discovered in the husk, such as saponins, flavonoids, coumarins, sterols, and so on [8, 14–16]. Among them, the rich barrigenol type triterpenoid saponins are the typical bioactive ingredients and mainly responsible for producing neuroprotective effect. Depending on the presence of hydroxy groups at C-15/C-16/C-21/C-22, the

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barrigenol type triterpenoid saponins in the husks could be categorized into barringtogenol C, R1-barrigenol and 16-deoxybarringtogenol C [8, 15]. The existing studies are mostly focused on the protective effect produced by a R1-barrigenol type triterpenoid saponin named xanthoceraside in the husks, including its pharmacodynamics [11, 12], pharmacokinetics [17], and mechanism [3–5, 7, 13], probably because its higher content in the husks extract [18]. In comparison, little attention has been brought to the research on other barrigenol type triterpenoid saponins. In our previous study [8], however, it was found out that a significant protective effect was exerted on A β _{25–35} induced PC12 cell death by not only xanthoceraside, but also two 16-deoxybarringtogenol C type triterpenoid saponins (compound 2 and the isomer of compound 5). Moreover, the two 16-deoxybarringtogenol C type triterpenoid saponins exhibited a less significant level of cytotoxicity than xanthoceraside at higher concentrations. Considering the synergistic reaction of the multiple components in the husks, it was presumed that the 16-deoxybarringtogenol C type triterpenoid saponins were also contributory to the protective effect of the husks on cognitive function. Nevertheless, there are still few pharmacokinetics studies focusing on the 16-deoxybarringtogenol C type triterpenoid saponins in the husks so far. In order to develop fruit-husks resources as the adjuvants and/or health food products intended to protect cognitive function, pharmacokinetics study is considered as a crucial link that deserves more attention than it has. Since the pharmacokinetics study of xanthoceraside has already been conducted [17], our focus is placed exclusively on the pharmacokinetics study of the five bioactive 16-deoxybarringtogenol C type triterpenoid saponins with the potential of neuroprotection.

In our previous study on the content quantitation of 16-deoxybarringtogenol C type triterpenoid saponins in the husks by High performance liquid chromatography-mass spectrometry (HPLC-MS), we found that these saponins exhibited the highest response in positive ion mode with $[M+2Na]^{2+}$ ions [19]. However, the stability of $[M+2Na]^{2+}$ ions made it difficult to obtain stable fragment ions. Thus, we used selected ion mode (SIM) instead of monitoring detection mode (MRM) to do the detection, and received an acceptable results. So in this study, a simple, sensitive, and reliable high-performance liquid chromatography-tandem mass spectrometry method in SIM was proposed for determining five 16-deoxybarringtogenol C type triterpenoid saponins in rat plasma after the oral administration of *X. sorbifolia* husks extract (XSE). The method was fully validated for its selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability. This study is expected to contribute a critical basis to the determination and clinical application of 16-deoxybarringtogenol C type triterpenoid saponins, which is valuable for the further development of the *X. sorbifolia* husks resource.

EXPERIMENTAL

Materials and reagents

The husks of *X. sorbifolia* were collected from Chifeng city, Inner Mongolia, China, which were identified by associated

professor Dong Wang in School of Pharmacognosy of Shenyang Pharmaceutical University in China. The five compounds (1–5) with purity of 98.1, 95.5, 96.5, 90.3, and 91.2% were all isolated from the husks by our group and their chemical structures were unambiguously identified by comparing the experimental NMR and HR-MS data with the previous reports [18, 19]. The chemical structures of the five compounds were shown in Fig. S1, and their names were provided in Table S1 in the supplementary material.

HPLC-grade methanol, acetonitrile, and formic acid were purchased from Fisher Scientific (USA). HPLC-grade ethyl acetate and isopropanol were obtained from Shandong Yu Wang Chemical Reagent Factory (Shandong, China). The DD water was provided by Wahaha Co., Ltd. (Hangzhou, China).

Preparation of *X. sorbifolia* husks extract (XSE) for oral administration

The powder of dried husks (~200 g) was extracted by refluxing 2 h with 70% ethanol for three times. Combined and filtered the solution, which were then evaporated under reduced pressure. The concentrate was redissolved in 0.5% sodium carboxymethylcellulose (CMC-Na) to obtain the *X. sorbifolia* husks extract (XSE) gastric soup at a concentration of 3.33 g/mL.

Collection of plasma samples

Six healthy male Sprague Dawley rats (180–220 g) (Certificate No. SCXK2010-0001) were obtained from Liaoning Changsheng Biotech Co. Ltd. (Liaoning, China). The rats were housed under controlled conditions (temperature: 21 ± 2 °C; relative humidity: $50 \pm 10\%$) with a natural light-dark cycle to acclimate for at least one week with free access to food and water. Before intra gastric dosing of XSE by a stomach tube (50 g/kg), the rats were fasted for at least 4 h but had free access to water. Approximately 0.3 mL of the blood samples were collected by orbital sinus bleeding in heparinized 0.5 mL Eppendorf tubes at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h after intra gastric dosing. Plasma was obtained by centrifuging at 12,000 rpm for 5 min and stored at -80 °C until analysis. Pharmacokinetic parameters were determined using the Drug and Statistics (DAS) software (version 2.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China) with a non-compartmental model. The animal study was carried out following the Animal Experimentation Guidelines of Shenyang Pharmaceutical University, and the protocol was approved by the Animal Ethics Committee of the institution.

Preparation of standard and quality control samples

The mixed standard stock solution of compounds 1–5 were prepared in methanol at the concentration of 2, 40, 2.5, 1, 5 μ g/mL, respectively, and the concentration of internal standard (IS) was 250 ng/mL. A series of standard solutions were obtained by diluting the stock solution with methanol. Calibration samples were prepared by adding the series standard solutions (20 μ L) and IS stock solutions (20 μ L) to blank rat plasma (200 μ L) to obtain concentrations of 20, 40, 200, 400,

1,000, and 2,000 ng/mL for compound 1, 400, 800, 4,000, 8,000, 20,000, and 40,000 ng/mL for compound 2, 25, 50, 250, 500, 1,250, and 2,500 ng/mL for compound 3, 10, 20, 100, 200, 500, and 1,000 ng/mL for compound 4, 50, 100, 500, 1,000, 2,500, and 5,000 ng/mL for compound 5 and 250 ng/mL for IS. Quality control (QC) samples were prepared independently at concentrations of 40, 250, and 1,600 ng/mL for compound 1, 800, 5,000, and 32,000 ng/mL for compound 2, 50, 312.5, and 2,000 ng/mL for compound 3, 20, 125, and 800 ng/mL for compound 4 and 100, 625, and 4,000 ng/mL for compound 5 using the same method as for the calibration samples. The stock solutions and working solutions were all stored at 4 °C until use.

Plasma sample preparation

Before analysis, the plasma samples were thawed to room temperature. An aliquot of 40 µL of IS solution was added to 200 µL of collected plasma sample. The solution was then extracted with 1 mL mixture of ethyl acetate and isopropanol (1:1, v/v) following with vortex-mixing for 5 min and centrifuging at 12,000 rpm for 5 min. The supernatant was evaporated to dryness at 30 °C under a stream of nitrogen. The residue was redissolved in 100 µL of methanol by vortex-mixing for 3 min, then centrifuged at 12,000 rpm for 5 min. Finally, a 5 µL of supernatant was injected into the HPLC-MS system for analysis.

Instruments, parameters, and conditions

The analysis was performed on a Prominence™ LC-20A HPLC system tandem an AB Sciex 4000 QTRAP™ MS system. Chromatographic separation was achieved on a Phenomenex C18 column (250 mm × 4.6 mm, 5.0 µm) (Phenomenex, USA) protected by a high pressure column pre-filter (2 mm) (Shimadzu, Japan) at 30 °C. The mobile phase system consisted of phase A (0.05% formic acid) and B (acetonitrile). The gradient elution program with run time of 20 min was shown as follows: 0–6 min, 32–33% B; 6–15 min, 33–40% B; 15–16 min, 40–32% B and kept constant for 4 min. The flow rate was set at 1.0 mL/min with 30% of the eluent being split into the inlet of the mass spectrometer.

An electro-spray ionization (ESI) source was used for mass spectrometric detection in positive ion mode. Target ions were monitored $[M+2Na]^{2+}$ at m/z 698.40 for compounds 1, 2, and 3, $[M+2Na]^{2+}$ at m/z 617.30 for compound 4, $[M+2Na]^{2+}$ at m/z 719.30 for compound 5, $[M+Na]^+$ at m/z 803.45 for IS using the SIM. After optimization, the source parameters were set as follows: curtain gas, 20 psi, nebulizer gas, 50 psi, turbo gas, 50 psi, ion spray voltage, 5,500 V, and temperature, 500 °C. Data acquiring and processing were performed using analyst software (version 1.6, AB Sciex).

METHOD VALIDATION

The method was fully validated according to the International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) guidelines [20, 21] for its

selectivity, linearity, the lower limit of quantification (LLOQ), accuracy, precision, recovery, and stability.

The selectivity of the method was evaluated by analyzing blank rat plasma obtained from six different rats to exclude the potential interferences at retention time for the compounds 1–5 and IS.

The calibration curves were established by plotting the peak area ratios of compounds 1–5 to IS versus the actual concentrations of the compounds 1–5 by weighted ($1/x^2$) least-squares linear regression. The acceptance criterion of a calibration curve was a correlation coefficient (r) of 0.99 or better and the REs for each point should be within $\pm 15\%$ ($\pm 20\%$ for the LLOQ). LLOQ was defined as the lowest concentration of compounds 1–5 in the calibration curve with a signal-to-noise ratio higher than 10.

The intra/inter-day precision and accuracy were determined by analyzing five replicates at three different QC levels on three sequential days. The precision and accuracy were expressed by relative standard deviation (RSD) and relative error (RE), respectively. The criteria for acceptability of the data included RE within $\pm 15\%$ and RSD less than 15%.

Extraction recovery and matrix effect for compounds 1–5 were assessed by the same set of spiked QC samples. The recovery was determined by comparing the analytical response of processed samples with those of post-processed samples spiked with compounds 1–5. The matrix effect was measured by comparing the response of post-processed samples spiked with compounds 1–5 with those unprocessed samples in reconstitution solution. The RSD of extract recovery and matrix effect at each concentration should be less than 15%.

The stability was evaluated by determining triplicates of QC samples under three conditions. Pre-treatment stability was evaluated after the exposure of untreated QC samples at room temperature (25 °C) for 4 h. Post-treatment stability was determined by using QC samples stored in the auto-sampler at 4 °C for 12 h. And for freeze-thaw stability, QC samples were conducted through three freeze-thaw cycles.

RESULTS AND DISCUSSION

Method validation

Typical chromatograms of the blank rat plasma, the blank rat plasma spiked with compounds 1–5 and IS, the rat plasma sample obtained 1 h after oral administration of XSE were shown in Fig. 1. Obviously, no endogenous interference was observed at retention time of compounds 1–5 or the IS.

The regression equations, correlation coefficient (r) and linear range of compounds 1–5 in plasma were listed in Table 1. Linearity performed well since correlation coefficients (r) were higher than 0.99. Meanwhile, the LLOQ were 20, 400, 25, 10, 50 ng/mL respectively for the five analytes and the REs for each point were all within $\pm 20\%$. As the results shown, LLOQs appears as an indicative value for the sensitivity of the method for analysis, which were sufficient for the pharmacokinetics study.

Fig. 1. Typical SIM chromatograms of blank plasma (A), plasma spiked with compound 1–5 at 500 ng/mL and IS at 400 ng/mL (B), and plasma samples obtained 2 h after an oral administration of the extract of XSE (C)

Table 1. The calibration parameters for compounds 1–5 in plasma using the established HPLC-ESI-MS method

Analyte	Liner range (ng/mL)	Slope	Intercept	Regression coefficient	QC1 concentration (ng/mL)	QC2 concentration (ng/mL)	QC3 concentration (ng/mL)
Compound 1	20–2000	0.00990	0.05475	0.9943	40	250	1,600
Compound 2	400–40,000	0.00221	0.4352	0.9952	800	5,000	32,000
Compound 3	25–2,500	0.00620	0.01071	0.9946	50	312.5	2000
Compound 4	10–1,000	0.00182	−0.001970	0.9955	20	125	800
Compound 5	50–5,000	0.00264	0.02149	0.9959	100	625	4,000

The detailed results of intra- and inter-day accuracies and precisions were showed in Table 2. Deviations of the accuracies (RE) and variations (RSD) for within day and between days were lower than 16.5%, which met the required criteria. It demonstrated this assay have good accuracy and precision.

The extraction recovery of compounds 1–5 was presented in Table 2. It was found that extraction recoveries ranged from 73.1 to 81.0% for compound 1, 83.0–84.8% for compound 2, 79.4–88.1% for compound 3, 80.7–83.7% for compound 4 and 72.4–86.6% for compound 5 in spiked rat plasma with a maximum RSD of 9.3%. The results indicated that the extraction approach employed in the present work gave reproducible recovery for the compounds 1–5. The

matrix effect for compounds 1–5 was illustrated in Table 3. It changed within a specified range in biological samples with RSD less than 12.9%, indicating that there was no obvious matrix effect for compounds 1–5 in the plasma samples for determination.

The stability of the analytes in rat plasma during 4 h at room temperature, during three freeze–thaw cycles and over 12 h in an auto-sampler agreed with ICH and FDA guidance [20]. The result was shown in Table S2 in the supplementary material. The concentrations of the analytes in rat plasma under different storage conditions were $100 \pm 15\%$ of the QC levels, demonstrating that five compounds have good stability under these conditions.

Table 2. Summary of recovery, matrix effect, accuracy and precision of compounds 1–5 in plasma

Analytes	Concentration (ng/mL)	Intra-day RSD % ^a	Inter-day RSD % ^a	Accuracy RE%	Recovery (% mean $\pm$ SD) ^b	Matrix effect (% RSD) ^c
Compound 1	20	12.7	11.1	2.5	–	–
	40	3.8	12.2	–3.0	74.87 $\pm$ 4.03	3.0
	250	8.5	9.3	4.3	71.28 $\pm$ 3.82	3.9
	1,600	10.0	4.7	2.8	81.0 $\pm$ 2.8	6.3
Compound 2	400	0.6	11.4	–3.9	–	–
	800	3.3	4.1	8.2	83.0 $\pm$ 1.7	1.3
	5,000	2.9	6.1	7.6	83.0 $\pm$ 3.4	6.0
	32,000	5.2	9.2	3.9	84.8 $\pm$ 4.7	5.0
Compound 3	25	7.5	16.5	–0.6	–	–
	50	11.9	8.12	2.4	83.8 $\pm$ 7.8	5.8
	312.5	7.1	3.2	3.3	88.1 $\pm$ 1.7	7.9
	2000	5.8	4.1	–4.2	79.44 $\pm$ 3.86	4.3
Compound 4	10	4.3	12.3	–0.9	–	–
	20	5.0	1.7	–11.0	81.9 $\pm$ 4.2	12.9
	125	4.9	6.8	–3.9	83.7 $\pm$ 3.7	3.0
	800	3.9	3.9	–6.1	80.7 $\pm$ 2.4	7.4
Compound 5	50	13.3	11.3	–1.1	–	–
	100	4.0	8.2	–12.3	86.6 $\pm$ 3.5	7.9
	625	2.8	3.6	–10.2	82.6 $\pm$ 6.4	2.8
	4,000	2.8	8.1	–6.6	72.38 $\pm$ 5.94	8.2

^aRelative standard of derivation (RSD), where $n = 6$.

^bRecovery (%) = (A/B $\times$ 100) %. The data was presented as average of six independent determinations, where $n = 6$. A: the responses of spiked plasma samples after extraction. B: the responses from standard QC solution.

^cMatrix effect: IS-normalized MF = (A/B $\times$ 100) %. The data was presented as average of six independent determinations, where $n = 6$. A: the MF of the analytes. B: the MF of the IS at QC levels; MF (matrix factor) was calculated by the ratio of peak area of post extracted blank matrix spiked with QC solutions and peak area without matrix.

Table 3. Mean pharmacokinetic parameters (mean $\pm$ SD) after oral administration of XSE to rats ($n = 6$)

	Compound-1	Compound-2	Compound-3	Compound-4	Compound-5
C_{max} (ng/mL)	298.4 $\pm$ 221.3	3,786 $\pm$ 2,438	704.5 $\pm$ 579.0	299.1 $\pm$ 255.4	2,726 $\pm$ 1,249
T_{max} (h)	1.25 $\pm$ 0.67	1.25 $\pm$ 0.67	1.38 $\pm$ 0.63	1.38 $\pm$ 0.63	1.46 $\pm$ 0.68
$T_{1/2}$ (h)	2.92 $\pm$ 0.57	5.52 $\pm$ 1.75	2.48 $\pm$ 0.62	2.95 $\pm$ 0.94	2.34 $\pm$ 0.81
$AUC_{(0-t)}$ (ng* h/mL)	1,031 $\pm$ 4,940	1.260 $\times 10^4 \pm 0.4080 \times 10^4$	2,479 $\pm$ 1,387	1,121 $\pm$ 9,410	0.9502 $\times 10^4 \pm 0.1995 \times 10^4$
$AUC_{(0-\infty)}$ (ng* h/mL)	1,118 $\pm$ 4,950	1.575 $\times 10^4 \pm 0.4380 \times 10^4$	2,594 $\pm$ 1,374	1,190 $\pm$ 9,280	0.9858 $\times 10^4 \pm 0.199 \times 10^4$

Pharmacokinetic study

The developed HPLC-ESI-MS method was applied to the pharmacokinetic study in healthy rats. The plasma samples with analytes concentration above the upper limit of quantitation were diluted with blank rat plasma. The mean plasma concentration-time curve after oral administration of XSE was shown in Fig. 2. The main pharmacokinetic parameters were summarized in Table 3. In this study, the behaviors of compounds 1–5 were similar. For isomers (compounds 1–3), the $t_{1/2}$ value of compound 2 was 5.52 ± 1.75 , longer than other compounds, indicating that the drug could distribute in the target tissue for longer time to exert effect. The double peak phenomena of compounds 1–5 possibly resulted from the entero-hepatic recirculation, gut microbiota metabolism, mutual transformation of metabolism, double-site absorption, and intestinal efflux [22]. Nevertheless, further studies should be conducted to confirm these findings in the future. The pharmacokinetic parameters above would provide significant indications to clinical application of the husks resource.

The MTT assays on $A\beta_{25-35}$ induced PC12 cells of compound 2 and the isomer of compound 5 had been published in our previous study [8], and the result of compound 4 were shown in Fig. S2 in the supplementary materials. The weaker cytotoxicity of 16-deoxybarringtonol C type triterpenoid saponins probably due to the presence of sugar groups at C-28 and the absence of angeloyl groups at C-21 and/or C-22 [8]. Since compound 1, 2, and 3 were isomers, and the only change is the position of the angeloyl group on the sugar groups at C-28, it was plausible that compounds 1 and 3 also had the protection on cognitive dysfunction in terms of the structure-activity relationship. The structure of compound 5 is very similar to compound 2, however, the effect of the acetyl group at C-21 on its activity need to be further explored. According to Serum Pharmacochimistry of Traditional Chinese Medicines (SPT) theory, for most bioactive constituents via oral administration, they should be first absorbed into blood, and then exerted therapeutic effect after metabolism and distribution [23]. Thus, it is plausible that the

Fig. 2. Mean plasma concentration-time profile of compound 1–5 after oral administration of the XSE

five 16-deoxybarringtonol C type triterpenoid saponins were potential bioactive components in the husks contributing to its protection on cognitive function. Currently, there were few research on the pharmacokinetics study of 16-deoxybarringtonol C type triterpenoid saponins in the husks. Our study could provide a valuable reference of the XSE on the absorption, distribution, metabolism, and excretion (ADME) in vivo, which were very useful on the clinical use of the husks resource.

CONCLUSION

This is the first pharmacokinetic study of five 16-deoxybarringtonol C type triterpenoid saponins in vivo following the oral administration of XSE by the HPLC-ESI-MS method. The five saponins detected in this study were believed to be responsible for the protection of the husks extract on cognitive dysfunction by MTT assays. However, due to their complex structures, it is difficult for them to be absorbed into blood, and their low content makes them harder to be detected. The other characteristic of compounds 1–5 was that they exhibited the highest response of $[M+2Na]^{2+}$ ions in positive ion mode without stable fragment ions. Thus, a simple, sensitive, and highly accurate HPLC-MS method in SIM instead of MRM was applied to determine the saponins in the plasma of rats, which could meet all requirements in bioanalysis. The study would provide some valuable references to clinical application of the fruit husks resource.

Conflict of interest: The authors declared that they have not conflicts of interest.

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SUPPLEMENTARY MATERIAL

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