Simultaneous determination of six triterpenoid saponins in beagle dog plasma by UPLC-MS/MS and its application to a pharmacokinetic study after oral administration of the extract of the Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. leaves

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ABSTRACT

A rapid and simple ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) method was developed and validated for simultaneous determination of six analytes from the Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. leaves (ESL) in beagle dog plasma for the first time, including 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside-29-hydroxy oleanolic acid, 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside-29-hydroxy oleanolic acid, 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-30-norlean-12,20-(29)-dien-28-olic acid, ciwujianoside E, guaianin N, and eleutheroside K. The chromatographic separation was performed using an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) using a gradient elution way with a mobile phase of acetonitrile-water containing 0.1% formic acid. Analytes were detected on a triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source with multiple reaction monitoring (MRM) mode. Calibration curves were all linear (r ≥ 0.9933) over the concentration range. The mean extraction recoveries and matrix effect of analytes and I.S. were ranged from 80.26% to 98.32% and from 91.27% to 111.67%, respectively. The intra-day and inter-day precision were ranged from 2.20% to 14.81%, and the accuracy range was 1.60–14.60%. The analytical method was successfully applied for the pharmacokinetic characteristics of the six analytes in beagle plasma after oral administration of ESL extracts. The T½ of six analytes was more than 3.09 ± 0.78 h.

KEYWORDS

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim., Pharmacokinetics, Triterpenoid saponins, UPLC-MS/MS

1. INTRODUCTION

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. leaves (ESL) has been officially recorded as an official health function drug by the China Food and Drug Administration, it was documented in the ancient Chinese medical book “Rihuazi Materia Medica”. And they are widely distributed in northeast China, Japan, northeast Russia. ESL are used for spleen and lung qi deficiency, physical weakness, loss of appetite, lung and kidney deficiency,
chronic cough and asthma, waist and knee pain, heart and spleen deficiency, insomnia, dreaminess [1–3] in aspect of Traditional Chinese Medicine (TCM). ESL was often produced as dietary supplement and tea. The tea of ESL has been used to treat chronic diseases like insomnia and diabetes [4, 5]. The young leaves of ESL have been used as a kind of edible vegetable and abortion for more than thousands of years.

ESL is mainly rich in triterpene saponins [6–11], flavonoids [12] and organic acids [13]. Saponin glycosides is its main active ingredient. In the in-depth development of ESL, many researchers have studied more on saponins active ingredients. In the preliminary study, we studied its main chemical components and pharmacological effects. Six triterpene saponins were isolated from ESL in preliminary research, including 3-O-α-L-rhamnopyranosyl-1→2)-α-L-arabinopyran Fruitsidase-29-hydroxyoleanolic acid (1), 3-O-β-D-glucopyranosyl-1→2)-α-L-arabinopyranosidase-29-hydroxyolean Acid (2), 3-O-β-D-glucopyranosyl-1→2)-α-L-arabinopyranosyl-30-norlean-12,20 (29) -dien-28-olic acid (3), eleutheroside E (4), guaiin N (5) and eleutheroside K (6). Among them, 4 has activities in treating diabetes, in aspect of deficiency, insomnia, dreaminess [1–3]. 6 has been reported to had weak inhibitory effect on nitric oxide production by lipopolysaccharide (LPS)-activated macrophage 264.7 anti-tumor effects [16–17], and anti-HIV, and anti-bacterial activity against pseudomonas and brine shrimp toxicity [18]. 6 has been reported to possess antispasmodic [19], anti-leishmanicidic [20], pro-apototic and anti-cancer activities [21]. In particular, 6 has no cytotoxic effect [22], but it could reverse chemotherapy resistance of breast cancer cells by reducing the formation and releases of D/exo, reversing docetaxel resistance [23]. Little research has been done on 1, 2, and 3. To the best of our knowledge, these six triterpenoid saponins have not been clinically used as drugs, nor have their metabolism been studied in vivo. Pharmacokinetics studies on ESL active ingredients would be helpful to study the efficacy and toxicity of ESL. At present, there are no pharmacokinetic studies on saponins from ESL have been reported, mainly because the molecular weight of saponins is large and it is difficult to detect in plasma [24]. In this study, a highly sensitive, novel method for simultaneously determination of the six triterpenoid saponins in Beagle dog plasma was established by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

2. EXPERIMENTAL

2.1. Chemicals and reagents

The reference standards for 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosidase-29-hydroxy oleanolic acid (1), 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosidase-29-hydroxy oleanolic acid (2), 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-30-norlean-12,20 (29)-dien-28-olic acid (3), eleutheroside E (4), guaiin N (5), eleutheroside K (6) were isolated from ESL, in pre-experiment, and based on the UV, NMR, MS, HPLC analysis, the chemical structures were identified. All purities were more than 98.0%. Butylparaben as internal standard (I.S.) (NO. 3801-2010, purity >98%) was purchased from the Tianjin Kermel (Tianjin, China). All HPLC-grade chemical reagents (methanol, acetonitrile, formic acid, and acetic acid) were purchased from Thermo Fisher Scientific (Shanghai, China). Analytical-grade dichloromethane, isopropylalcohol and ethyl acetate n-butanol was purchased from Xilong Scientific (Guangdong, China). The Millipore Milli-Q plus system (Millipore Inc, Molsheim, France) was used to prepare deionized water, which was prepared from Watsons water (Guangzhou, China).

2.2. Equipment and UPLC-MS/MS conditions

Chromatographic separation was carried out on Waters ACQUITY UPLC system (Waters Inc. Milford, MA, USA) using an ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column. The gradient mobile phase system was composed of solution A (0.1% formic acid-water) and solution B (0.1% formic acid - acetoni-trole), which was distributed at a flow rate of 0.4 mL min⁻¹. The gradient elution was 50%–50% B at 0.0–0.5 min, 50%–70% B at 0.5–1.0 min, 70%–70% B at 1.0–1.5 min, 70%–90% B at 1.5–4.0 min, 90%–90% B at 4.0–5.0 min. The elution time was 5.0 min. The temperature of column and the auto-sampler were at were 40°C and 10°C, respectively. The auto-sampler takes about 10 μl sample for injection.

UPLC system was connected to an AB Sciex 4000 triple quadrupole tandem mass spectrometer (AB Sciex Inc, Toronto, ON, Canada). The ion source was electrospray ionization (ESI) interface (Framingham Inc, MA, USA). The quantitative analyte detection was performed by using multiple reaction monitoring (MRM) in negative mode. The transitions of precursor-product ion at m/z 749.4→471.3 for 1, 765.6→603.6 for 2, 733.4→571.4 for 3, 717.4→493.4 for 4, 749.9→587.7 for 5, 733.3→455.3 for 6, and 193.0→92.0 for I.S., respectively. The parameters of the source were set as follows: ionspray voltage 4500 V; capillary temperature 450°C; ion source gas 1 (GS1) flow 50 psi; ion source gas 2 (GS2) flow 50 psi; curtain gas flow 10 psi, the dwell time 100 ms. The declustering potential (DP) was 205.6 V for 1, 189.48 V for 2, 228.68 V for 3, 183.15 V for 4, 217.18 V for 5, 191.59 V for 6, and 98.32 V for butylparaben, respectively. The nebulizing gas was Liquid nitrogen (N2), the drying gas was nitrogen (N2). The operating conditions are the flow rate of 400 L h⁻¹ and the temperature of 40°C.
microporous resin column (H2O – 30% EtOH – 60%). Finally, 60% of the ethanol component were drying after lyophilized. Then the extract of ESL was obtained.

The contents of the six compounds in ESL were quantified by using a HPLC-ELSD method in order to calculate the administration. The concentrations of the six analytes were 14.03 μg g⁻¹ for 1, 26.83 μg g⁻¹ for 2, 6.76 μg g⁻¹ for 3, 9.38 μg g⁻¹ for 4, 15.56 μg g⁻¹ for 5, and 34.20 μg g⁻¹ for 6, respectively.

2.4. Preparation of calibration and quality control samples
A mixed stock solution was prepared by dissolving the mixed standard with methanol, and the standard working solutions of concentration that was 210.2 μg mL⁻¹ for 1, 201.6 μg mL⁻¹ for 2, 250.0 μg mL⁻¹ for 3, 220.2 μg mL⁻¹ for 4, 225.6 μg mL⁻¹ for 5 and 230.0 μg mL⁻¹ for 6. Standard working solutions were acquired by further diluting the stock solution with methanol. Internal standard solution (200.0 ng mL⁻¹) was obtained through further dilution of stock solution (600.0 ng mL⁻¹) with methanol.

The final concentrations of 1 were 0.6570–210.2 ng mL⁻¹; 0.6300–201.6 ng mL⁻¹ for 2; 0.7815–250.0 ng mL⁻¹ for 3; 0.6880–220.2 ng mL⁻¹ for 4; 0.7050–225.6 ng mL⁻¹ for 5; 0.7190–230.0 ng mL⁻¹ for 6. Quality control (QC) samples at four concentration levels: LLOQs, LQCs, MQCs and HQCs were prepared in the same way, 0.6570, 1.314, 8.04, and 168.16 ng mL⁻¹ for 1, 0.6300, 1.260, 80.64 and 161.28 ng mL⁻¹ for 2, 0.7815, 1.563, 100.0 and 200.0 ng mL⁻¹ for 3, 0.6880, 1.376, 88.08, and 176.16 ng mL⁻¹ for 4, 0.7050, 1.410, 90.24, and 180.48 ng mL⁻¹ for 5, and 0.7190, 1.438, 92.00, and 184.00 ng mL⁻¹ for 6. All the standard working solutions were prepared on site, and stored at -20°C.

2.5. Preparation of samples
Six male beagle dogs (body weight 9.95 ± 0.77 kg, age 4 ± 1.0 years old) were purchased from Shenyang Kangping Experiment Animal Research Institute. The animal license number was SCXK 2014–0003. The six beagle dogs were raised in the Experimental Animal Center of Heilongjiang University of Chinese medicine (Harbin, China). All the experimental behaviors and operations were approved by the Institutional Ethics Committee of Heilongjiang University of Chinese medicine (Harbin, China). The experimental operation was carried out according to the Laboratory Animals Guidelines for Ethical Review of Welfare (GB/T 35,892–2018). The purpose of this study was to compare the experimental methods of 6 target analytes after oral ESL in beagle dogs and to investigate their pharmacokinetic characteristics. In the future, we will expand the sample size for further research and confirmation of the results. Dogs were reared under appropriate conditions, with relative humidity of 60 ± 5%, room temperature of 22 ± 2°C and light conditions consistent with circadian rhythm. While acclimating to the environment, dogs were kept on ordinary clean grade diet for 3 days, fasted for 12 h and free water before administration. 2 mL of blood sample was drawn from forearm vein at following time points (0, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 12.00 and 24.00 h) after oral administration of ESL into 5 mL heparinized polythene tubes. The blood samples were immediately centrifuged at 3500 RPM for 25 min at 4°C. The separated plasma were stored at −20°C until analysis. The plasma concentration time data for the six analytes in plasma were calculated by the software DAS 2.1 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) in non-compartmental mode. The results were expressed as mean ± SD.

The 100 μL plasma samples were placed in 5 mL centrifuge tubes, and then 250 μL methanol, 50 μL acetonitrile, and 100 μL I.S. solution were added into the centrifuge tubes. The solution obtained from the previous step were vortex-mixed for 2 min, followed by ultrasonic oscillation for 1 min, ethyl acetate-isopropanol (1:1) was added, and then vortexed for 2 min. The solution was then centrifuged at 1300 RPM for 15min and the supernatant was collected into 1.5 mL centrifuge tubes, evaporated at 40°C under a pure nitrogen stream and the residue was reconstituted in 100 μL of methanol. After swirling for 1 min, the mixture was transferred into a 1.5 mL centrifuge tube, centrifuged at 13,000 RPM at 4°C for 10 min, and finally the supernatant was filtered through a 0.22 μm membrane.

2.6. Method validation
The developed bioanalytical method was validated according to the principles of Guidance for Industry Bioanalytical Method Validation by the US Food and Drug Administration (FDA), considering selectivity, linearity, precision, accuracy, extraction recovery, matrix effect, and stability [25].

2.6.1. Selectivity. By comparing the plasma samples of six different batches of blank dog, the blank plasma was spiked with the analytes and I.S., and the chromatogram of the plasma samples was collected for 2.5 h after the ESL extract was taken orally for comparison. Endogenous interference was analyzed in the plasma of the blank beagle dogs, followed by spiking with I.S. for the interference of I.S.

2.6.2. Linearity and lower limits of quantification (LLOQ). The linearity of calibration curve was determined by plotting the peak area ratio (S/N) versus concentration on the calibration curve at which the signal-to-noise ratio (S/N) ≥ 10.

2.6.3. Precision and accuracy. The intra-day precision and accuracy were evaluated at LLOQ, LQC, MQC, HQC concentration levels of the analytes in six replicates on the same day and analyzed continuously for three days. Similarly, the inter-day precision and accuracy of the analyte were measured at four concentration levels over six replicates over three consecutive days.
The precision was expressed as a percentage relative SD (RSD%), which was the ratio of the SD to the arithmetic mean of the measurement results. The precision was illustrated as a relative error (RE), which should be within ±15%.

2.6.4. Extraction recovery and matrix effect. The extraction recoveries were assessed by comparing the mean peak areas of analytes obtained from QC samples with those obtained from pure reference standards spiked in post-extracted blank plasma at LQC, MQC, HQC levels. The matrix effect was assessed by comparing the peak area of an analyte added to the extracted supernatant with that of a standard solution containing the analyte of the same concentration.

2.6.5. Stability. The stability of the analytes was evaluated in dog plasma at LQC, MQC, HQC concentrations levels in dog plasma under the following conditions (n = 6). The short-term stability of blood samples was investigated by storing them at room temperature (25°C) for 4 h. And the long-term stability was evaluated for kept at −20°C for 4 weeks. In addition, the freeze-thaw stability was tested after three complete freeze/thaw cycles (−20°C–25°C) on consecutive days. And the post-preparation stability was evaluated after placed in the auto-sampler (10°C) for 24 h before analyze. It can be considered that the analytes were stable when the deviation less than ±15%. The calibration curve of the newly developed standard is used to test all the tested QC samples.

3. RESULTS AND DISCUSSION

3.1. Optimization of the UPLC-MS/MS condition

MS/MS parameters were optimized for determination of the analytes and I.S. According to the previously reported, negative mode was applied for triterpenoids ion monitoring. The positive and negative mode were investigated to obtain the precursor and product ion, the response of the analytes observed in the negative ionization mode was higher than that in positive ionization mode. Therefore, the negative ion mode was selected for the MS/MS detection. And the response of the analytes observed in the negative mode was stable. The electrospray ionization (ESI) source was used as ionization interface. The optimization of MS/MS parameters were accomplished by the manual turning tool to obtain optimal sensitivity of product ion of analytes and I.S. The optimized mass transitions ion pairs of the precursor to production were ascertained for MRM. All the products and qualifier ions were selected basing on the stability and high ion response. The optimized mass transitions ion pairs and parameters are listed in Table 1. MS/MS fragmentation patterns of the six analytes and I.S. are illustrated in Fig. 1.

3.2. Optimization of chromatography

Two organic phases consist of acetonitrile and methanol were tested, respectively. The responses of analytes and I.S. were higher with acetonitrile-water as mobile phase than that methanol-water through a series of trials. The 0.1% formic acid can improve the peak shape and the mass response. To eliminate the undesirable crosstalk effects and achieve a complete chromatographic resolution, we shorten the analysis time and increased the peak capacity within 3.0 min [26]. The flow rate and column temperature were adjusted to acquire a great resolution with no crosstalk observed. Finally, the separation was carried out by gradient elution at column temperature 40°C consisting of acetonitrile-water added 0.1% formic acid at a flow rate of 0.4 mL min⁻¹. There was no carry-over effect detected above method.

3.3. Optimization of extraction method

In order to obtain high extraction recovery and little endogenous interference at the retention time, several solvents such as methanol, acetonitrile, methanol-acetonitrile were investigated by precipitation of protein (PPT) in dog plasma. PPT with methanol-acetonitrile (v/v, 5:1) were to increase the extraction rate of the six triterpenoid saponins and reduce the matrix effect. It was illustrated that methanol-acetonitrile for PPT produced the best extraction recovery for all the analytes and I.S. Finally, plasma samples were prepared with methanol-acetonitrile (v/v, 5:1) precipitation for lowest noise interference level.

3.4. Selection of I.S

An ideal I.S should be a stable isotope-labeled compound or structurally similar compound [27]. Butylparaben was selected as the I.S. because of its high mass spectral response and good chromatographic peak shape. Furthermore, the

| Table 1. Precursor/production pairs and parameters for MRM of analytes |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Analyte | Ionization mode | Precursor Ion (m/z) | Product Ion (m/z) | Qualifier Ion (m/z) | DP (V) | CE (V) |
| 1 | negative | 749.4 | 471.3 | 587.3 | −205.60 | −59.14 |
| 2 | negative | 765.6 | 603.1 | 585.4 | −189.48 | −57.34 |
| 3 | negative | 733.4 | 571.4 | 587.7 | −228.68 | −55.06 |
| 4 | negative | 717.4 | 439.3 | 571.1 | −183.15 | −58.75 |
| 5 | negative | 749.9 | 587.7 | 471.3 | −217.18 | −59.35 |
| 6 | negative | 733.3 | 455.3 | 587.2 | −191.59 | −61.10 |
| I.S. | negative | 193.0 | 92.0 | 135.9 | −98.32 | −33.74 |
Fig. 1. Product ion mass spectra of 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside-29-hydroxy oleanolic acid (A), 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside-29-hydroxy oleanolic acid (B), 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-30-norlean-12,20(29)-dien-28-olic acid (C), eleutheroside E (D), guaianin N (E), eleutheroside K (F), and I.S. (G)
retention time of butylparaben was stable in the above liquid phase conditions, and little interference was detected in plasma.

3.5. Method validation

3.5.1. Selectivity. The selectivity of the method was evaluated with plasma in contrary to endogenous plasma matrix from six beagle dogs. Under the developed UPLC-MS/MS conditions, all the analytes could be separated and quantitatively determined at LLOQ and MQC levels. The retention time of six analytes and I.S. was 0.98 min for 1, 0.85 min for 2, 2.01 min for 3, 2.23 min for 4, 2.40 min for 5, 2.65 min for 6, and 1.90 min for I.S., respectively. There was no significant interference observed at the retention time of the analytes and I.S. The Respective chromatograms of blank plasma, plasma sample spiked with the analytes and I.S., and the plasma sample gained from dog after oral administration of the total saponin of ESL are shown in Fig. 2.

3.5.2. Linearity and lower limit of quantification. The regression equation, correlation coefficients and linearity ranges for the six analytes are shown in Table 2. The linear calibration ranges were 0.6570–210.2 ng mL⁻¹ for 1, 0.6300–201.6 ng mL⁻¹ for 2, 0.7815–250.0 ng mL⁻¹ for 3, 0.6880–220.2 ng mL⁻¹ for 4, 0.7050–225.6 ng mL⁻¹ for 5, 0.7190–230.0 ng mL⁻¹ for 6, respectively. The correlation

![Fig. 2. Respective MRM Chromatograms of 1 (1), 2 (2), 3 (3), 4 (4), 5 (5), 6 (6) and I.S. (7): (a) blank plasma, (b) blank sample spiked with the analytes at LLOQ and I.S., (c) blank sample spiked with the analytes at MQC and I.S., (d) sample from beagle dogs at 2.5 h after oral administration of ESL extract](image-url)
coefficients were high than 0.9933. The results revealed that there was great correlation between the peak area ratio of analytes to I.S. and concentration within the linearity ranges. The LLOQs was 0.6570 ng mL\(^{-1}\) for 1, 0.6300 ng mL\(^{-1}\) for 2, 0.7815 ng mL\(^{-1}\) for 3, 0.6880 ng mL\(^{-1}\) for 4, 0.7050 ng mL\(^{-1}\) for 5, 0.7190 ng mL\(^{-1}\) for 6.

### 3.5.3. Precision and accuracy.

The intra-day and inter-day precisions and accuracies were evaluated by determination of QC samples at LLOQ, LQC, MQC, HQC levels in six replicates on a same day and on three consecutive validation days, respectively. The results of precision and accuracy of all the analytes are shown in Table 3. The intra-day and inter-day precisions ranged from 2.20 to 14.81% and 2.21–14.80%, respectively. The accuracies were from −1.60% to 14.60%. The assay values on precision and accuracy were within the acceptable range (<15%). These results demonstrated an excellent precision and accuracy for the quantification of the six analytes in plasma.

#### 3.5.4. Extraction recovery and matrix effect.

The extraction recoveries and matrix effects of the six analytes at three QC concentration levels are displayed in Table 4. The extraction recoveries of the six analytes from beagle dog plasma were 80.26–98.32% at three QC levels (LQC, MQC, HQC), and the mean matrix effect in plasma ranged from 93.39% to 111.67% for the six analytes at three QC levels. The averaged extraction recovery and matrix effect of I.S. were 98.40% and 91.27%, respectively. The results indicated that the recovery rate of PPT procedure in dog plasma was satisfactory for all analytes and there no endogenous substances obstruct the determination of the analytes.

#### 3.5.5. Stability.

The stability of the six analytes were evaluated under four storage conditions at three QC levels. The results were presented in Table 5. The results indicated that the plasma samples were stored at room temperature (25°C) for 4h, at −20°C for one month, and in the automatic sampler (10°C) for 24h. The plasma samples were stable during

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**Table 2.** The regression equations, linear ranges, LLOQs of analytes

| Compound | Linear Regression Equation | Linear Range (ng mL\(^{-1}\)) | r  | LLOQ (ng mL\(^{-1}\)) |
|----------|-----------------------------|-----------------------------|----|---------------------|
| 1        | \(Y = 2.02 \times 10^{-4} X + 4.0 \times 10^{-4}\) | 0.6570–210.2 | 0.9956 | 0.6570 |
| 2        | \(Y = 5.01 \times 10^{-4} X + 9.0 \times 10^{-4}\) | 0.6300–201.6 | 0.9962 | 0.6300 |
| 3        | \(Y = 6.22 \times 10^{-3} X - 4.2 \times 10^{-3}\) | 0.7815–250.0 | 0.9987 | 0.7815 |
| 4        | \(Y = 1.10 \times 10^{-3} X - 1.3 \times 10^{-3}\) | 0.6880–220.2 | 0.9981 | 0.6880 |
| 5        | \(Y = 7.04 \times 10^{-4} X - 1.2 \times 10^{-3}\) | 0.7050–225.6 | 0.9956 | 0.7050 |
| 6        | \(Y = 1.20 \times 10^{-3} X - 3.0 \times 10^{-4}\) | 0.7190–230.0 | 0.9933 | 0.7190 |

**Table 3.** Precision and accuracy for the determination of the 6 triterpenoid saponins in dog plasma (n = 6)

| Compound | Spiked Concentration (ng mL\(^{-1}\)) | Mean ± SD (ng mL\(^{-1}\)) | Intra-day Precision RSD (%) | Inter-day Precision RSD (%) | Accuracy RE (%) |
|----------|------------------------------------|---------------------------|-----------------------------|-----------------------------|-----------------|
| 1        | 0.6570                             | 0.6600 ± 0.32             | 6.30                        | 8.20                        | −10.40          |
|          | 1.314                              | 1.5000 ± 0.12             | 7.60                        | 12.00                       | 14.60           |
|          | 0.6300                             | 0.6300 ± 0.10             | 4.20                        | 5.30                        | 10.20           |
|          | 0.7815                             | 0.7855 ± 0.42             | 3.21                        | 2.10                        | −3.00           |
|          | 1.563                              | 1.7220 ± 0.27             | 14.81                       | 12.3                        | 10.40           |
|          | 100.00                             | 96.59 ± 5.63              | 2.20                        | 3.80                        | −1.60           |
|          | 200.00                             | 199.21 ± 1.19             | 6.4                         | 6.90                        | −3.80           |
|          | 0.6880                             | 0.6900 ± 0.19             | 4.50                        | 6.20                        | 2.10            |
|          | 1.376                              | 1.2601 ± 0.19             | 13.5                        | 14.1                        | −1.70           |
|          | 88.08                              | 86.26 ± 11.41             | 7.90                        | 8.90                        | 3.70            |
|          | 176.16                             | 175.12 ± 3.68             | 13.5                        | 10.80                       | −2.00           |
|          | 0.7050                             | 0.7100 ± 0.50             | 5.10                        | 8.70                        | −3.40           |
|          | 1.410                              | 1.5455 ± 0.12             | 6.80                        | 11.70                       | 6.80            |
|          | 90.24                              | 90.01 ± 5.14              | 5.30                        | 11.60                       | 1.90            |
|          | 184.08                             | 177.89 ± 2.99             | 5.40                        | 7.70                        | −2.20           |
|          | 0.7190                             | 0.7201 ± 0.11             | 6.00                        | 5.03                        | −2.30           |
|          | 1.438                              | 1.6012 ± 0.13             | 8.00                        | 8.10                        | 11.40           |
|          | 92.00                              | 90.47 ± 5.66              | 12.20                       | 5.30                        | 7.10            |
|          | 184.00                             | 180.25 ± 5.70             | 6.30                        | 6.30                        | −1.70           |
three-free thaw cycles, and the accuracy was in the range of −14.82–14.91%. The results were within the acceptance limits of ±15%.

3.6. Pharmacokinetic study

The validated method was successfully applied to the pharmacokinetic study in plasma after the oral administration of the ESL extract at a dose of 0.84 g kg⁻¹ to the beagle dog. The dog dosage used was converted from the human dosage recorded in the Chinese pharmacopoeia [28]. The typical plasma concentration-time profiles of the analytes are shown in Fig. 3 (see Fig. 1). The pharmacokinetic parameters including the half-time (T₁/₂), the maximum plasma concentration (C_max), the time to reach the maximum concentrations (T_max), elimination rate constants (Kₑ), area under concentration-time curve (AUC₀-∞, AUC₀-∽∞) are shown in Table 6.

The T_max of 1, 2, 3, 4, 5 and 6 were 1.70 ± 0.36, 2.51 ± 0.05, 2.30 ± 0.24, 2.39 ± 0.17, 2.66 ± 0.02, and 2.50 ± 0.41 h, respectively. The T_max ranged 1.70–2.66 h, which indicated the absorption of the components (except 1) was relatively slow. The reason might be due to the molecular masses of the six saponins larger than 700 Da (the favorable value) [29]. The T_max required for 2 and 5 was significantly more than for other analytes, perhaps related to their substituent’s arabinose and glucose, and the T_max of 6 was slightly less than the T_max of 2 and 5. Perhaps related to the substitution of two arabinoses. The T₁/₂ of 1, 2, 3, 4, 5 and 6 were 3.27 ± 0.78, 3.12 ± 1.20, 3.09 ± 0.78, 3.97 ± 0.35, 4.01 ± 3.62, and 3.90 ± 1.17 h, respectively. Both T_max and T₁/₂ of 5 and 6 were higher than other analytes, which demonstrated that low absorption of 5 and 6. By the way, this may be related to their Angular methyl of C-20. The former results indicated that the metabolism of the six analytes were different in vivo. The difference might be related with different substituents groups’ chemical structure. The differences of the C_max of six analytes maybe concerned with their contents in ESL. The AUC₀-∞ ranged from 173.16 ± 55.72 to 518.19 ± 181.29 ngh⁻¹mL⁻¹, and the AUC₀-∽∞ ranged from 140.88 ± 29.75 to 436.31 ± 65.34 ngh⁻¹mL⁻¹, which corresponded to

Table 4. The averaged extraction recoveries and matrix effects of the 6 triterpenoid saponins and I.S. in dog plasma (n = 6)

| Compound | Concentration (ng mL⁻¹) | Recovery | Matrix effect |
|----------|-------------------------|----------|--------------|
|          | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| 1        | 1.314      | 98.32 | 14.74 | 98.99 | 2.87 |
|          | 84.08      | 94.69 | 4.26 | 97.86 | 4.49 |
|          | 168.16     | 90.98 | 10.68 | 105.18 | 14.82 |
| 2        | 1.260      | 97.99 | 4.62 | 95.63 | 0.75 |
|          | 80.64      | 96.77 | 14.58 | 93.39 | 1.15 |
|          | 161.28     | 84.06 | 6.68 | 102.83 | 4.85 |
| 3        | 1.563      | 92.64 | 13.85 | 95.05 | 5.92 |
|          | 100.00     | 86.61 | 7.22 | 110.12 | 9.86 |
|          | 200.0      | 88.58 | 11.52 | 107.92 | 7.12 |
| 4        | 1.376      | 97.23 | 3.70 | 93.11 | 0.44 |
|          | 88.08      | 87.81 | 4.12 | 95.09 | 2.01 |
|          | 176.16     | 90.92 | 10.36 | 100.37 | 6.52 |
| 5        | 1.410      | 94.21 | 5.37 | 94.22 | 2.89 |
|          | 90.24      | 93.69 | 3.75 | 93.69 | 4.39 |
|          | 180.48     | 89.90 | 14.97 | 111.67 | 14.16 |
| 6        | 1.438      | 80.26 | 13.15 | 97.83 | 2.74 |
|          | 92.00      | 82.01 | 7.82 | 111.40 | 7.03 |
|          | 184.00     | 83.04 | 13.14 | 104.41 | 12.48 |
| I.S.     | 200.00     | 98.40 | 11.06 | 91.27 | 3.71 |

Table 5. Stability of the 6 triterpenoid saponins in dog plasma (n = 6)

| Compound | Concentration (ng mL⁻¹) | Stability (% RE) |
|----------|-------------------------|------------------|
|          | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| 1        | 5.07      | 2.21 | 2.65 | 3.65 |
|          | 84.08 | −11.99 | 4.04 | 5.64 | 10.94 |
|          | 168.16 | 10.3 | 4.02 | 3.47 | 4.29 |
| 2        | 2.24      | −0.355 | −0.56 | 4.87 |
|          | 80.64 | 13.31 | −3.00 | −7.41 | 4.87 |
|          | 161.28 | 12.58 | 1.04 | 3.42 | −5.59 |
| 3        | 6.82      | 5.96 | 3.52 | 4.08 |
|          | 100.00 | −9.21 | −12.06 | −0.34 | −3.75 |
|          | 200.00 | −7.6 | −14.49 | −13.49 | −3.39 |
| 4        | 1.376     | 14.18 | 1.15 | 6.637 | 1.015 |
|          | 88.08 | −7.24 | −14.94 | 8.51 | 4.64 |
|          | 176.16 | −3.64 | −13.36 | 1.322 | 3.34 |
| 5        | 1.410     | 5.13 | 2.52 | 12.57 | 14.64 |
|          | 90.24 | −13.11 | 14.88 | 13.82 | 4.58 |
|          | 180.48 | −8.14 | −13.74 | −8.38 | −14.82 |
| 6        | 1.438     | 3.58 | 14.91 | 13.05 | 4.397 |
|          | 92.00 | 10.017 | −10.31 | 13.66 | 10.04 |
|          | 184.00 | 0.224 | −6.62 | −14.43 | −2.76 |
administration dosage and excretion. Both $T_{1/2}$ and $\text{AUC}_{0-\infty}$ increased slightly, inferring that the six saponins have a similarly slow distribution and slow elimination process, and it is likely to accumulate in vivo [30]. These results would be sense to further studies on the pharmacokinetics, toxicity, and pharmacology of ESL and promote research into the efficacy of the TCM herb in clinical therapeutic studies.

**Table 6.** Main pharmacokinetic parameters of the six analytes after oral administration of ESL to beagle dogs (mean ± SD, n = 6)

| Analytes | $C_{\text{max}}$ (ng mL$^{-1}$) | $T_{\text{max}}$ (h) | $T_{1/2}$ (h) | $\text{AUC}_{0-1}$ (ngmL$^{-1}$h) | $\text{AUC}_{0-\infty}$ (ngmL$^{-1}$h) |
|----------|--------------------------------|----------------------|---------------|---------------------------------|-----------------------------------|
| 1        | 53.78 ± 7.14                   | 1.70 ± 0.36          | 3.27 ± 0.72   | 229.08 ± 42.40                  | 253.43 ± 55.93                    |
| 2        | 38.72 ± 12.59                  | 2.51 ± 0.05          | 3.12 ± 1.20   | 157.25 ± 40.17                  | 173.16 ± 55.72                    |
| 3        | 50.01 ± 7.59                   | 2.30 ± 0.24          | 3.09 ± 0.78   | 181.81 ± 15.02                  | 198.93 ± 25.46                    |
| 4        | 49.13 ± 15.65                  | 2.39 ± 0.17          | 3.89 ± 0.35   | 193.83 ± 70.01                  | 222.15 ± 84.28                    |
| 5        | 30.63 ± 5.07                   | 2.66 ± 0.02          | 4.01 ± 3.62   | 140.88 ± 29.75                  | 180.82 ± 6.45                     |
| 6        | 79.57 ± 5.66                   | 2.50 ± 0.41          | 3.90 ± 1.17   | 436.31 ± 65.34                  | 518.19 ± 181.29                   |
4. CONCLUSION

A rapid and selective UPLC-MS/MS method was developed for the simultaneous determination of six compounds of ESL in beagle dog plasma. The beagle dogs were used as experimental animals. It was successfully investigated the pharmacokinetics of the six triterpenoid saponins of ESL in beagle plasma. The pharmacokinetics parameters of the six analytes showed a process of similarly slow absorption and quick elimination in the body. The results were helpful for the clinical application of ESL, and provided a foundation for further research on the pharmacology mechanisms and toxicology of ESL.

Data availability: The data used to support the findings of this study are included within the manuscript and are available from the corresponding author upon request.

Conflicts of interest: The authors declare no conflict of interest.

Author’s contributions: Zhibin Wang conceived the experiments; Feng Cao and Yaodan Chang designed and performed the experiments; Yaodan Chang analyzed the data; Zhibin Wang contributed reagents/materials/analysis tools; Yaodan Chang and Zhenyue Wang wrote the paper. Chunjuan Yang, Haixue Kuang Polish the paper. All authors read and approved the final manuscript.

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