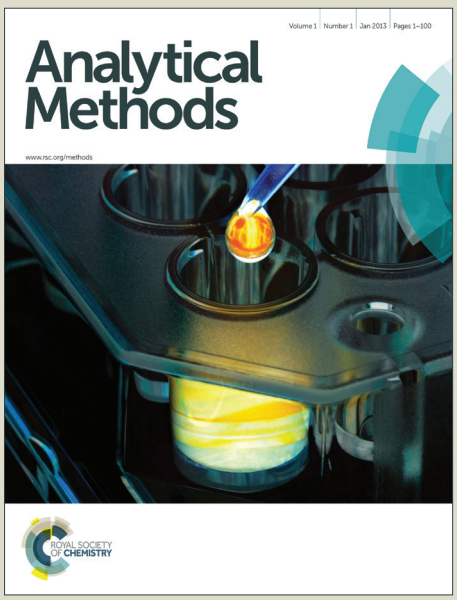
Analytical Methods

Accepted Manuscript

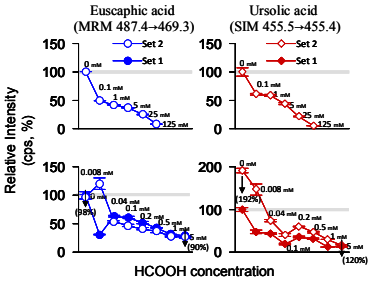


ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Table of contents

Colour graphic:



Text: Formic acid (0.1-0.2‰) in mobile phase overcame the matrix effects and increased the sensitivity of MS detection of euscaphic acid.

Sensitive and cost-effective LC–ESI–MS/MS method for quantitation of euscaphic acid in rat plasma using optimized formic acid concentration in mobile phase

Feng Chen, [a,b,†] Hai-Long Li, [a,b,†] Yin-Feng Tan, [a,b] Wei-Yong Lai, [a,b] Zhen-Miao Qin, [a,b] Hong-Die Cai, [a] Yong-Hui Li, [a,b] Jun-Qing Zhang, [a,b,*] Xiao-Po Zhang[a,b,*]

a. School of Pharmacy, Hainan Medical University, Haikou 571101, China

b. Hainan Provincial Key Laboratory of R&D of Tropical Herbs, Hainan Medical University, Haikou 571101, China

† These authors contributed equally to this work.

* Corresponding authors at: School of Pharmacy, Hainan Medical University, Haikou 571101, China. Tel.: +86 898 66895337; Fax: +86 898 66893460.
E-mail addresses: jqzhang2011@163.com (J.-Q. Zhang), xiaopozhang@yahoo.com (X.-P. Zhang).

ABSTRACT

Euscaphic acid, a triterpene acid, exists ubiquitously in medicinal plants and demonstrates various pharmacological activities. This active compound is often used as a marker compound for quality control. Hitherto, the pharmacokinetic (PK) information was relatively scarce; therefore, it remains open to question whether the euscaphic acid reaches the target sites in the body at concentrations high enough for the claimed biological effects. A robust analytical method is prerequisite for obtaining enough PK information of euscaphic acid, which is useful for interpreting its pharmacological effects. In this study, we developed and validated a rapid liquid chromatographic tandem mass spectrometric (LC-MS/MS) for the measurement of euscaphic acid in rat plasma. The rat plasma samples were precipitated with acetonitrile and the resulting supernatants were separated using a 4-min pulse gradient method on a Synergi Fusion-RP C_{18} column (4 μm, 2.0 mm i.d. × 50 mm). Ursolic acid was used as internal standard for quantification of euscaphic acid. Deprotonated euscaphic acid and its internal standard were generated at negative electrospray ionization (ESI) mode and their precursor-to-product ion pairs (m/z 487.4 → 469.3 and 455.5 → 455.4, respectively) were used for measurement. Notably, the commonly used concentration of formic acid (HCOOH; 1‰ and 5‰, v/v) in mobile phase seriously suppressed the signal intensity, but this mobile phase additive at much lower concentration level (0.1‰ and 0.2‰) could overcome the matrix effects and therefore increased the sensitivity of MS detection of euscaphic acid. The newly developed bioanalytical assay which possessed favorable accuracy and precision with lower limit of quantification of 2.0 ng/mL and was successfully applied to PK studies in rats. The experimental strategies presented herein may be helpful for measurement of other triterpene acids in biological matrices.

Keywords: Euscaphic acid; Ursolic acid; HCOOH; LC–ESI–MS/MS; PK

Introduction

Euscaphic acid (2α, 3α, 19α-trihydroxyurs-12-en-28-oic acid, $C_{30}H_{48}O_5$), a triterpene acid, was firstly isolated from the medicinal plant, *Euscaphis japonica* Pax (Staphyleaceae), [1] which is a deciduous shrub or small tree distributed in southeastern central China, southwestern Japan and northern Taiwan. [2] Parts of the plant have been used by an ethnic minority in Guizhou province, China, to treat detumescence and analgesia.[3] Additionally, euscaphic acid was chosen as a marker compound for quality control of *Potentilla discolor*,[4] *Callicarpa macrophyll*,[5] *Folium Eriobotryae* [6] and *etc*. Pharmacological researches both *in vitro* and *in vivo* have revealed that euscaphic acid has a variety of biological activities. For example, euscaphic acid exhibited significant protein tyrosine phosphatase 1B inhibitory activity, with half maximal inhibitory concentration (IC_{50}) values ranging from 3.5 to 54.8 μM.[7] Euscaphic acid showed inhibitory effect against enzymes involved in DNA replication and the IC_{50} values were 61 μM for calf DNA α-polymerase and 108 μM for rat DNA β-polymerase, respectively. This compound could prevent the growth of BALL-1 cancer cells and the median lethal dose (LD_{50}) value was 48 μM.[8] Compared to α-arbutin, euscaphic acid demonstrated an obvious decrease in intracellular melanin content in B16-F10 cells and in culture media melanin (IC_{50} 14.1 μM).[9] Euscaphic acid concentration-dependently (50–200 μM) reduced the production of nitric oxide, prostaglandin E_2, tumor necrosis factor-α and interleukin-1β induced by LPS in RAW 264.7 macrophages.[10] Euscaphic acid was found to possess antimicrobial activity with minimum inhibitory concentration values in the range of 3.2–205 μM.[11] This triterpene acid could also inhibit atherosclerosis and xanthoma in low-density lipoprotein receptor knockout mice after oral administration of euscaphic acid at a dose of 10 mg/kg for 24 weeks.[12] Euscaphic acid was orally administered to Sprague-Dawley rats (30 mg/kg) for 7 d prior to injecting carrageenan and the significant anti-inflammatory effects were observed.[13] Overall, reported concentrations of euscaphic acid exerting *in vitro* pharmacological activities range from 3.2 to 205 μM, while PK assessments of euscaphic acid have been relatively

scarce.[4] Therefore, it remains open to question whether the euscaphic acid or its metabolites exists in the body at concentrations high enough for the claimed biological effects.

PK information of natural products could be used to bridge the gap between phytochemistry and pharmacology.[14] Body exposure to the bioactive constituents of a botanical drug is a crucial determinant of its drug response and therefore the efficacy and safety.[15,16] In order to understand the PK behavior of euscaphic acid, it is critical to develop robust analytical methods to deal with various biological samples (*e.g.*, tissues and body fluids such as plasma, bile and urine). Up to now, there has only been one assay which is a HPLC–UV based method for the determination of euscaphic acid for the quality control of *Prunellae Spica*[17] and another assay is available with LC–ESI–MS method for the quantification of euscaphic acid in plasma samples of normal and diabetic rats after oral administration of *P. discolor* extract.[4] In the former assay, the LOD value and LOQ value was 290 ng/mL and 880 ng/mL, respectively. Although a relatively lower LOQ value (2.5 ng/mL) was obtained in the latter one, a longer run time (18 min) and a multiple-step biosample preparation procedure were needed. Thus, these validated methods could not facilitate fast and efficient PK evaluation for euscaphic acid.

LC–MS/MS has been widely used for the assay of drugs and their metabolites in biological matrices due to its high sensitivity, selectivity and rapid rate of analysis. However, successful use of LC–MS/MS requires fully understanding the principles of various sample extraction procedures and of both chromatography and mass spectrometry.[18,19] For example, ursolic acid, another triterpene acid, could not be collided into fragments when collision energy was lower than 40 eV, or no dominant product ions were detected if collision energy was higher than 50 eV, which indicated that routine multiple reaction monitoring (MRM) with different parent and product ion was not suitable for ursolic acid quantitation.[20] In order to achieve the goals of "faster, better and cheaper" for biosamples analysis, analyte-dependant method development and validation is required.

The major goal of this study was to develop and validate a fast LC–MS/MS

method to determine the plasma concentration of euscaphic acid and to apply this method to analyze samples obtained from a single intravenous (i.v.) and oral (p.o.) PK study in Sprague-Dawley (SD) rats. Notably, we found that the inclusion of HCOOH (0.008–125 mM) into the mobile phase suppressed signal intensity of the euscaphic acid and ursolic acid (used as internal standard, IS). However, this electrolyte modifier could also increase the sensitivity and overcome the matrix effects for both analytes using appropriate concentrations.

Experimental

Chemicals and materials

Euscaphic acid was separated from the root of *Rosa cymosa* using various column chromatographies particularly the semi-preparative HPLC method in our lab. The chemical structure of euscaphic acid was confirmed by analyzing its NMR data and comparing with reported literature. Its purity (>98%) was determined by HPLC equipped with a UV detector and an Aglient Eclipse XDB-C_{18} column (5μm, 4.6 mm i.d. × 250 mm). Reference standard of ursolic acid (purity > 98%, lot no. MUST-13020602) was purchased from Chengdu MUST Bio-Technology Co., Ltd. (Chengdu, China). HPLC-grade methanol and acetonitrile were products of Tedia Company Inc. (Fairfield, OH, USA). HPLC-grade HCOOH was purchased from Aladdin Industrial Inc. (Shanghai, China). Purified water was prepared using the Millipore system (Millipore, Bedford, MA, USA). The other chemical reagents of analytical grade or better were obtained from Hainan YiGao Instrument Co., Ltd (Haikou, China). Chromatographic columns including Luna 5 μm C_{18} (2) (in-house No. 1#), Kinetex 2.6 μm XB-C_{18} (No. 2#), Synergi 4μm Fusion-RP C_{18} (No.3#), Synergi 4μm Hydro-RP C_{18} (No. 4#) and Gemini 3μm C_{18} (No.6#) were purchased from Guangzhou FLM Scientific Instrument Co., Ltd (Guangzhou, China).

LC-MS/MS analysis

The LC-MS/MS system consisted of an AB-SCIEX API 4000^{+} mass spectrometer (Toronto, Canada) interfaced via a Turbo V ion source with a Shimadzu Prominence UFLC chromatographic system (Kyoto, Japan), which is equipped with two LC-20AD pumps, a model DGU-20A3R degasser unit, a SIL-20A HT autosampler and a CTO-20A column oven. The AB-SCIEX Analyst software packages were used to control the LC–MS/MS system, as well as for data acquisition and processing.

Chromatographic separations of prepared samples were achieved using a Phenomenex Synergi Fusion-RP C_{18} column (4 μm, 2.0 mm i.d. × 50 mm) maintained at 40ºC, before which a 0.5-μm biocompatible inline filter (Upchurch Scientific, Oak

Harbor, WA, USA) was used. The LC mobile phase (delivered at 0.50 mL/min) included water containing 2.5 mM HCOOH for solvent A and methanol containing 2.5 mM HCOOH for solvent B. A specially designed "pulse gradient"[21] was performed with the gradient program as follows: 0–0.3 min at 0% B; from 0% B to 100% B in 0.01 min (0.31min) and maintained 2.7 min (0.31–3 min); from 100% B to 0% B in 0.01 min (3.01 min) and maintained 1 min (3.01–4 min).

The mass spectrometer was operated in the negative ESI ion mode with MRM mode for euscaphic acid and selected ion monitoring (SIM) mode for ursolic acid. The pneumatically nebulized ESI spraying was achieved by using inner coaxial nebulizer N_2 gas (GS1) of 45 psi through a TurboIonSpray probe, a high voltage of –4.5 kV applied to the sprayer tip and heated dry N_2 gas (GS2) of 55 psi at 600 ºC from two turbo heaters adjacent to the probe. To prevent solvent droplets from entering and contaminating the ion optics, a curtain N_2 gas of 35 psi was applied between the curtain plate and the orifice. The in-source collision gas (CAD) flow was set at level 12. The precursor-to-product ion pairs (Fig. 1) used for MRM of euscaphic acid and ursolic acid were m/z 487.4→469.4 and 455.4→455.3, respectively, with a scan time of 40 ms for each ion pair.

Stock and working solutions

Stock solutions (1 mg/mL) of euscaphic acid and IS were prepared in acetonitrile and then diluted to working solutions with acetonitrile. All solutions were stored at –40ºC. The stability of the stock solutions was confirmed by comparing measurements of freshly prepared stock solutions with stock solutions stored for at least 1 month.

Calibration and quality control samples

Appropriate volumes of working solutions were diluted in methanol, whereof 10 μL were added to 490 μL of blank plasma then diluted with blank plasma step by step, obtaining nine calibration standards at concentrations from 0.25 to 2000 ng/mL for euscaphic acid. Low, medium and high concentration quality controls (QC) for euscaphic acid were designed at 6, 60 and 600 ng/mL according to a pilot study. QC

samples were prepared from separate stock solutions in the same way as the calibration standards.

Sample preparation

Plasma samples were prepared using a protein precipitation method at a precipitant-to-plasma volume ratio of 3:1 (v/v). In brief, a 50 μL aliquot of thawed plasma sample was mixed with 150 μL of acetonitrile containing the IS (500 ng/mL). The mixture was mixed by vortex-shaking for 5 min and centrifuged at 13, 000 rpm for 10 min. Ten microliters of the resulting supernatant were directly applied for LC–MS/MS analysis.

Assay validation

Assay validation was carried out according to the US FDA guidance on bioanalytical method validation (http://www.fda.gov/cder/guidance/index.htm) to demonstrate that the newly developed bioanalytical method was reliable for the intended applications.

Pharmacokinetic study

All rat experiments were performed in accordance with the Institutional Animal Care and Use Committee at the Hainan Medical University (Haikou, China), as well as the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006). Female Sprague Dawley (SD) rats (200–230 g) were purchased from DongChuang Laboratory Animal Service Department (Changsha, China). The rats were maintained under controlled temperature of 24 ± 2°C and relative humidity of 60% ± 10% with a 12-h light/dark cycle. Commercial rat chow was available *ad libitum* except for an overnight fasting period before dosing. All rats had free access to water throughout the experimental period.

For the i.v. and p.o. administration, euscaphic acid was dissolved in a mixture containing 6% (v/v) PEG400, 9.8% (w/v) Tween-80 and 4.4% (v/v) ethanol to achieve a concentration of 1 mg/mL. Six female SD rats were divided into two groups randomly: one group was given a single p.o. dose of euscaphic acid at 10 mg/kg and the other group was given at a dosage of 1 mg/kg intravenously. Serial blood samples

(~ 0.3 ml each at 5, 15 and 30 min, 1, 2, 4, 6, 8, 10 and 24h post-dosing) were collected in heparinized tubes. The blood samples were centrifuged to obtain the plasma fractions that were frozen at –70ºC until analysis.

Plasma PK parameters were calculated by a noncompartmental method using the Kinetica 2000 software package (version 3.0; Innaphase Corp., Philadelphia, PA, USA). The maximum concentration in the concentration-time profile (C_{max}) and the time to reach that concentration (t_{max}) were observed values with no interpolation. The area under concentration-time curve up to the last measured time point ($AUC_{0\rightarrow t}$) was calculated by the trapezoidal rule. The $AUC_{0\rightarrow\infty}$ was generated by extrapolating the $AUC_{0\rightarrow t}$ to infinity. Results are expressed as the mean ± SD.

Results and discussion

Optimization of LC–MS/MS conditions

Positive and negative ionization ion modes were investigated and compared to obtain good specificity and sensitivity for euscaphic acid and IS determination. The responses at the negative ion mode were found to be more sensitive than those at the positive ion mode by infusing a 1 μg/mL standard stock solution of analyte and IS in methanol using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA). As shown in Fig. 1, the mass spectra for euscaphic acid and IS revealed peaks at m/z 487.4 and 455.5, respectively, as deprotonated molecular ions $[M-H]^-$. The product ion mass spectrum for euscaphic acid shows the formation of characteristic product ions such as $[M-H_2O-H]^-$ at m/z 469.4 and $[M-CO_2-H_2O-H_2O-H]^-$ at m/z 407.4. For IS compound, lower collision energy (-40 v) could not easily collide this compound into fragments; however, no dominant product ions were measured if collision energy was higher than -80 v. The fragment ion m/z 407.4 was a minor product ion for IS. The mass spectrum characteristics of IS was consistent with a recently published article.[20]

(Insert Fig. 1 here)

Optimization GS1 is helpful for the best signal stability and sensitivity. Meanwhile, the GS2 aids in the evaporation of solvent, which helps to increase the ionization of the sample. In this study, the precursor-to-product ion pairs used for MRM of euscaphic acid (487.4→469.4) and IS (455.4→407.4), as well as SIM of the analyte (487.4→487.3) and IS (455.5→455.4), were selected for further optimization in negative mode. The mass parameter optimization results for euscaphic acid and IS are shown in Fig. 2a and 2b, respectively. The peak areas of SIM mode for both analytes were higher than those of MRM mode. However, the baseline noise levels of the SIM mode were higher too (see section below). The response intensities of both analytes increased first and then decreased along with curtain gas value changing from 15 psi to 50 psi. The inflection point value was at 35 psi. Curtain gas flow controls the flow of gas to the Curtain Gas interface. It prevents ambient air and

solvent droplets from entering and contaminating the ion optics, while permitting direction of sample ions into the vacuum chamber by the electrical fields generated between the vacuum interface and the spray needle. As for Curtain Gas flow, one should maintain this parameter as high as possible without losing sensitivity. Therefore, the inflection point value (35 psi) was selected for both analytes' quantification. Similarly, the parameter of Gas 2 was set at 55 psi, especially according to the response intensity of SIM mode for IS. The in-source collision gas profiles of SIM mode for both analytes at different levels exhibited concave curves, while those of MRM mode were increasing lines. Overall, the optimized ion source parameters for euscaphic acid and IS were as follows: CAD at level 12, 35 psi for Curtain Gas flow, 45 psi for Gas 1, 55 psi for Gas 2, -4.5 kv for IonSpray voltage and 600°C for heater temperature. The peak areas under the optimized mass parameters were higher (3~4 folds) than those of data under commonly suggested conditions.

(Insert Fig. 2a and 2b here)

In this study, our task was to develop an approach which could lead us to the faster analysis for our application. The mobile phase including methanol/H_2O system and acetonitrile/ H_2O system were firstly tested. For euscaphic acid, the response and peak shape were comparable between the two tested systems. However, the intensity of IS was decreased significantly and the peak for 455.4→407.4 ion pairs almost could not be detected using acetonitrile/H_2O as mobile phases. Obviously, methanol/H_2O system stood successfully. And then, we developed a LC pulse gradient elution method (the bottom panel of Fig. 3a) for the direct analysis of supernatant which was prepared by acetonitrile precipitation of the plasma protein components. The gradient parameters included a 0.3-min start proportion segment (SPS, min, 0% methanol), a 2.7-min elution proportion segment (EPS, min, 100% methanol) and a 1-min column equilibrium segment (CES, min, 0% methanol). This pulse gradient elution resulted in band compression and enhanced the performance of the chromatography.[21]

Various approaches were developed to optimize performances in liquid

chromatography. Small particle size attributes to lower theoretical plate heights, shorter column lengths and higher optimum eluent velocities. High pressure drives fluid faster and can be used in conjunction with smaller particles and narrower columns (≤ 2.1 mm i.d.). High temperature reduces viscosity and thus pressure; allows fluid to move faster at same pressure; improves interphase mass transfer and can be combined with use of smaller particles.[22,23] In the present study, we investigated the influence of different columns (the bottom panel of Fig. 3b) on the performance of both analytes when the flow rate (0.5 mL/min) and temperature (40°C) were preset and fixed. Fully porous particles were introduced into the columns including 1#, 3#, 4# and 6# except for 2 # with core-shell particles. The particle size was varied from 2.6 to 5 μm while the column length is fixed at 50 mm.

(Insert Fig. 3a and 3b here)

As shown in Fig. 3a, the peak area and height of euscaphic acid increased and then decreased along with particle size increasing. The peak width widened slightly, on the contrary, the retention time got shorter. The results were almost the same for ursolic acid (Fig. 3b). Therefore, the column 3# was chosen as the optimum chromatographic column for the final assay of the two triterpene acids. In addition, the responses of euscaphic acid and IS under SIM mode were higher than those of values under MRM mode. However, the baseline noise levels were obviously higher under SIM mode than those of MRM mode (550 folds and 17 folds, respectively). Correspondingly, the response of euscaphic acid under SIM mode was 4.5-fold higher than that of MRM mode. The value for IS was 43 times. Thus, the SIM mode (*i.e.*, 455.5→455.4) was used to quantitative analysis for the IS compound.

Low concentration of HCOOH in mobile phase suppressed the analytes' signal, but overcame the matrix effects

It is well known that the ESI signal of analytes could be significantly affected by mobile phase additives, such as HCOOH, CH_3COOH and $HCOONH_4$, since the process of electrospray involves the conversion of the ions present in the solution to ions in the gas phase.[24] In general, adding mobile phase additive is a feasible way to

enhance sensitivity by changing the ionic form of the analytes in solution thereby improving ionization efficiency. The commonly used concentration is 1‰ or 5‰. However, Li *et al*. found that the low level of mobile phase $HCOONH_4$ (0.01‰) obviously increased the signal intensities for all test flavonoids versus experiments run with an $HCOONH_4$-free mobile phase, whereas the ionization was severely suppressed when the $HCOONH_4$ concentration was increased to the more traditional use of concentration at 1‰.[25] In the current study, we evaluated the impact of various concentrations of HCOOH in the MeOH/H_2O-based mobile phase on the responses of the euscaphic acid and ursolic acid. As shown in Fig. 4 (upper panel), inclusion of HCOOH (0.1 mM, 0.0038‰ v/v) in the mobile phase resulted in the ion suppression of both analytes spiked in the acetonitrile-precipitated rat plasma samples (500 ng/mL). When the HCOOH concentration was increased to 125 mM (5‰), the analytes' responses were inhibited almost completely compared with an HCOOH-free mobile phase. Whereafter, the HCOOH influence at lower concentration (0.008–5 mM) was assessed. The LC mobile phase containing 0.008 mM HCOOH showed increased analytes' responses in plasma matrix-matched samples (Set 2), whereas the signal intensities decreased in acetonitrile solution samples (Set 1). When the mobile phase did not contain any HCOOH, the absolute matrix effect was 98% and 192% for euscaphic acid and ursolic acid, respectively. However, these values changed into 391% and 312% at 0.008 mM HCOOH. Interestingly, the additive reduced the absolute matrix effect at 5 mM (0.2‰) and no significant matrix effect on both analyte and IS was observed (90% and 120%, respectively), although the overall signal intensity was inhibited.

(Insert Fig. 4 here)

The bottom panel of Fig. 4 demonstrates the influence of HCOOH concentration on the response of euscaphic acid at low concentration, *i.e.*, 1 and 2.5 ng/mL. Euscaphic acid in plasma matrix-matched sample almost could not be identified at 1 ng/mL (S/N=1) until the concentration increased to 2.5 ng/mL (S/N=7.4) when HCOOH-free mobile phase was used. However, presence of low concentration of

HCOOH in the MeOH/H_2O mobile phase (2.5 mM, *i.e.*, 0.1‰) dramatically increased the detectability of the euscaphic acid and decreased the matrix effects with S/N ratios of 6.2 and 15.3 at 1 and 2.5 ng/mL, respectively. Overall, the low level of mobile phase HCOOH suppressed the signal intensity, but overcame the matrix effects and therefore increased the sensitivity of MS detection of euscaphic acid.

Method validation

Linearity and lower limit of quantification

The calibration curve (Y=0.00049X+0.00395, weight coefficient $1/X^2$) was linear over the measured range of 2-2000 ng/mL for euscaphic acid with correlation coefficient of 0.994. The lower limit of quantification was 2 ng/mL (S/N>10), with a precision of 1.66% and accuracy of 99.4% for this compound.

Accuracy and precision of the assay

Intra- and inter-run precision and accuracy data are shown in Table 1. Accuracy, ranging from 88.7% to 98.1%, was well in line with the FDA guidance. Intra- and inter-batch deviations ranged from 3.88% to 8.85% and from 5.53% to 7.83%, respectively. Therefore, the precision and accuracy data were within the acceptable criteria and allowed the accurate analysis of the euscaphic acid in rat plasma.

(Insert Table 1 here)

Matrix effects and recovery

Matrix effects and extraction efficiencies were assessed in quintuplicate by comparing analyte peak areas of across three different sample sets.[25,26] In set 1, analytes were dissolved in matrix component-free solvent. In set 2, analytes were added into five different lots of post-extracted plasma from untreated rats. In set 3, analytes were added to untreated plasma and then extracted. The absolute matrix effect and extraction recovery were calculated as follows:

$$\text{Absolute matrix effect} = (\text{Mean peak area})_{\text{set 2}} / (\text{Mean peak area})_{\text{set 1}}$$

$$\text{Extraction efficiency} = (\text{Mean peak area})_{\text{set 3}} / (\text{Mean peak area})_{\text{set 2}}$$

This post-extraction spike method provides a quantitative understanding of the level of matrix effect observed for specific analytes.[27] As shown in Table 2, the

extraction recovery ranged from 86.4% to 90.4%. The average matrix effects at all measured concentrations were 87.9%–100%. The extraction recovery and matrix effects were all within the acceptable range.

(Insert Table 2 here)

Stability

The storage of plasma samples at room temperature for 4 h (pretreatment) according to the normal sample preparation duration did not alter signal responses of euscaphic acid (Table 3). Processed samples (post-treatment) were stable at auto-sampler room for 8 h, according to the time period needed for the assay of 105 samples. The accuracy between initial and final analysis were between 87.3% and 102%, with RSD ranging from 2.99% to 5.24%. Three free-thaw cycles did not influence the stability of euscaphic acid (mean variation < 15%). Thus, euscaphic acid was acceptably stable under the tested conditions.

(Insert Table 3 here)

Pharmacokinetic study

Our validated method was used to quantify plasma concentration of euscaphic acid after a single p.o. and i.v. administration of euscaphic acid to rats. The plasma concentration time profiles of euscaphic acid after i.v. and p.o. dosing in rats are shown in Fig. 5. The key PK parameters are summarized in Table 4.

(Insert Fig. 5 and Table 4 here)

Plasma euscaphic acid was detected up to 24 h and presented a bimodal profile around 0.083 h and 6–8 h after i.v. dosing (Fig. 5), with the mean maximum plasma concentration of 635 ng/mL (1.30 μM) and 80 ng/mL (0.16 μM), respectively . The mean $t_{1/2}$ value was 4.61 h. The mean $CL_{tot,p}$ value was 1.91 L/h/kg. The V_{SS} value of euscaphic acid (13.2 L/kg) was greater than the rat total body water by volume (0.67 L/kg),[28] suggesting that this triterpene acid might tend to bind to tissue components (*e.g.*, proteins, lipids), which usually happens for the compound with high lipophilicity. Similarly, euscaphic acid was monitored in all the rat plasma samples

for 24 h after p.o. administration. The plasma concentration-time curves were bimodal with the first peak concentrations occurring 5–15 min and the second ones appearing 4–8 h after dosing (Fig. 5). The mean systemic bioavailability of euscaphic acid after p.o. administration of the pure compound solution was around 40%. The double peaking phenomenon in the plasma concentration-time curves likely resulted from enterohepatic circulation of the euscaphic acid.[29]

Conclusion

In this study, we report the development and validation of a rapid method for measurement of euscaphic acid in rat plasma treated by a simple protein precipitation procedure. Mass spectrometric and chromatographic conditions were systematically optimized. Notably, the more traditional use concentration of HCOOH in mobile phase seriously suppressed the signal intensity, but this mobile phase additive at very low concentrations could overcome the matrix effects and therefore increased the sensitivity of MS detection of euscaphic acid. The newly developed bioanalytical assay was accurate and sensitive, and was successfully applied to a rat PK study of euscaphic acid. Further studies are needed to characterize the absorption, distribution, metabolite profiles, elimination pathways and systemic exposure-dose relationship of euscaphic acid.

Acknowledgements

This work was supported by Grant 81460629 from the National Science Fund of China, Grants ZDZX2013008-2, ZDZX2013008-3 and ZDXM 2014071 from the Hainan Science and Technology Major Project, Grants 812189, 813196 and 813188 from the Natural Science Fund of Hainan Province and Grant 2011BA101B07 from the National Science & Technology Pillar Program during the 12th Five-Year Plan Period of China. The work was also financially supported by Grants HY2012-013 and HY2012-006 from the Hainan medical university for Young scholars. We are also grateful to Dr. Chen Cheng, Dr. Xiu-Xue Li and Dr. Mei-Juan Li from Shanghai Institute of Materia Medica for screening relevant articles from reference lists.

Competing interests

There are no competing interests to declare.

References

1 K. Takahashi, S. Kawaguchi, K. Nishimura, K. Kubota, Y. Tanabe, *Chem. Pharm. Bull. (Tokyo)*, 1974, **22**, 650–653

2 L.J. Zhang, J.J. Cheng, C.C. Liao, H.L. Cheng, H.T. Huang, L.M. Kuo, Y.H. Kuo *Planta Med.*, 2012, **78**, 1584–1590

3 J.J. Cheng, L.J. Zhang, H.L. Cheng, C.T. Chiou, I.J. Lee, Y.H. Kuo, *J. Nat. Prod.*, 2010, **73**, 1655–1658

4 J.J. Li, Y. Li, M. Bai, J.F. Tan, Q. Wang, J. Yang, *Biomed. Chromatogr.*, 2014, **28**, 717–724

5 P. Pan, L.Y. Jia, Q.S. Sun, *Chin. J. Pharm. Anal.*, 2007, **27**, 841–843

6 X.P. Cai, S.L. Li, J.L. Hua, H. Lv, W.L. Li, J.M. Ju, *Chin. J. Exp. Tradit. Med. Formulae*, 2013, **19**, 84–87

7 D. Li, W. Li, K. Higai, K. Koike, J. Nat. Med. 2014, **68**, 427–431

8 C. Murakami, K. Ishijima, M. Hirota, K. Sakaguchi, H. Yoshida, Y. Mizushina, *Biochim. Biophys. Acta*, 2002, **1596**, 193–200

9 N.Y. Song, J.G. Cho, D. Im, D.Y. Lee, Q. Wu, W.D. Seo, H.C. Kang, Y.H. Lee, N.I. Baek, *Nat. Prod. Res.*, 2013, **27**, 2219–2223

10 I.T. Kim, S. Ryu, J.S. Shin, J.H. Choi, H.J. Park, K.T. Lee, *J. Cell Biochem.*, 2002, **113**, 1936–1946

11 Y. Zhang, F. Bao, J. Hu, S. Liang, Y. Zhang, G. Du, C. Zhang, Y. Cheng, *Planta Med.*, 2007, **73**, 1596–1599

12 Q. Zhang, Z. Chang, Q. Wang, Cardiovasc. *Drugs Ther.*, 2006, **20**, 349–357

13 H.J. Jung, J.H. Nam, J.Choi, K.T. Lee, H.J. Park, *Biol. Pharm. Bull.*, 2005, **28**, 101–104

14 C. Li, *Curr. Drug Metab.*, 2012, **13**, 491–493

15 F. Chen, L. Li, F. Xu, Sun Y., F.F. Du, Ma X.T., C.C. Zhong, X.X. Li, F.Q. Wang, N.T. Zhang, C. Li, *Br. J. Pharmacol.*, 2013, **170**, 440–457

16 F. Chen, H.L. Li, Y.F. Tan, Y.H. Li, W.Y. Lai, W.W. Guan, J.Q. Zhang, Y.S. Zhao, Z.M. Qin, *J. Pharm. Biomed. Anal.*, 2014, **97**, 166–177

17 M.K. Lee, Y.M. Ahn, K.R. Lee, J.H. Jung, O.S. Jung, J. Hong, *Anal. Chim. Acta*, 2009, **633**, 271–277

18 L. Li, D.D. Tian, F. Chen, J.L. Yang, K. Yu, Y. Sun, *Curr. Drug Metab.*, 2012, **13**, 1206–1212

19 M. Jemal and Y.Q. Xia, Curr. Drug Metab., 2006, **7**, 491–502

20 Y.Y. Xia, G.L. Wei, D.Y. Si, C.X. Liu, *J. Chromatogr. B*, 2011, **879**, 219–224

21 Y.F. Li, Y. Sun, F.F. Du, K.H. Yuan, C. Li, *J. Chromatogr. A*, 2008, **1193**, 109–116

22 P.W. Carr, D.R. Stoll, X. Wang, *Anal. Chem.*, 2011, **83**, 1890–1900

23 P.W. Carr, X. Wang, D.R. Stoll, *Anal. Chem.*, 2009, **81**, 5342–5353

24 Y. Liang, T.Y. Guan, Y.Y. Zhou, Y.N. Liu, L. Xing, X. Zheng, C. Dai, P. Du, T. Rao, L.J. Zhou, X.Y. Yu, K. Hao, L. Xie, G.J. Wang, *J. Chromatogr. A*, 2013, **1297**, 29–36

25 L. Li, S.P. Liang, F.F. Du, C. Li., *J. Am. Soc. Mass Spectrom.*, 2007, **18**, 778–782

26 F. Chen, H.L. Li, Y.F. Tan, W.W. Guan, Y.H. Li, J.Q. Zhang, *Chem. Cent. J.*, 2014, **8**, doi: 10.1186/1752-153X-8-2

27 B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, *Anal. Chem.*, 2003, **75**, 3019–3030

28 B. Davies and T. Morris, *Pharm. Res.*, 1993, **10**, 1093–1095

29 N.M. Davies, J.K. Takemoto, D.R. Brocks, J.A. Yáñez, *Clin. Pharmacokinet.*, 2003, **49**, 351–377

Fig. 1 Chemical structures and MS/MS spectra of euscaphic acid (bottom panel) and ursolic acid (IS, top panel).

Fig. 2 MS parameters optimization of euscaphic acid (a) and IS (b). For each MS/MS parameter optimization, the other normal LC-MS/MS conditions were used, including ion source parameters (collision gas, level 5; curtain gas, 35 psi; Gas I, 45 psi; Gas II, 55 psi; ionspray voltage, -4.5 kv; temperature, 550ºC) and LC parameters (flow rate, 0.50 mL/min; column oven temperature, 40 ºC).

Fig. 3 The influence of different columns on the LC performance of euscaphic acid (a) and IS (b) when the flow rate (0.5 mL/min) and temperature (40°C). The pulse gradient (bottom panel of Fig. 3a) parameters included a 0.3-min start proportion segment (SPS, min, 0% methanol), a 2.7-min elution proportion segment (EPS, min, 100% methanol) and a 1-min column equilibrium segment (CES, min, 0% methanol). The column parameters are shown in the bottom panel of Fig. 3b.

Fig. 4 Effects of different concentrations of HCOOH in the MeOH/H_2O-based mobile phase on the signal intensities of euscaphic acid and IS in negative ion ESI mode (top panel). The peak areas of the MS/MS signal are given relative to that obtained using an HCOOH-free mobile phase (gray line), which is set to 100%. The low level of mobile phase HCOOH overcame the matrix effects and improved the detectability of euscaphic acid (bottom panel).

Fig. 5 Plasma concentration-time profiles of euscaphic acid after a single i.v. (2 mg/kg, left panel) and p.o. (10 mg/kg, right panel) administration to rats.

Fig. 1

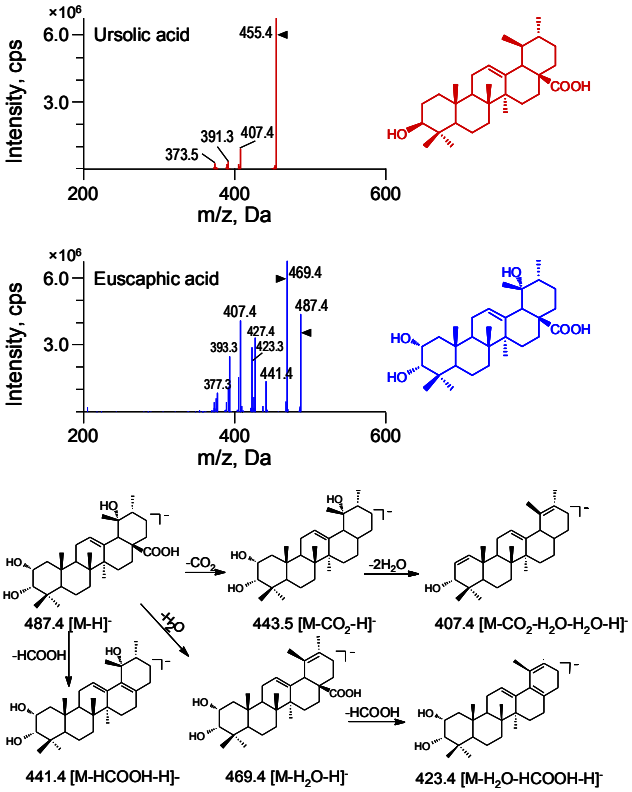


Fig. 2a

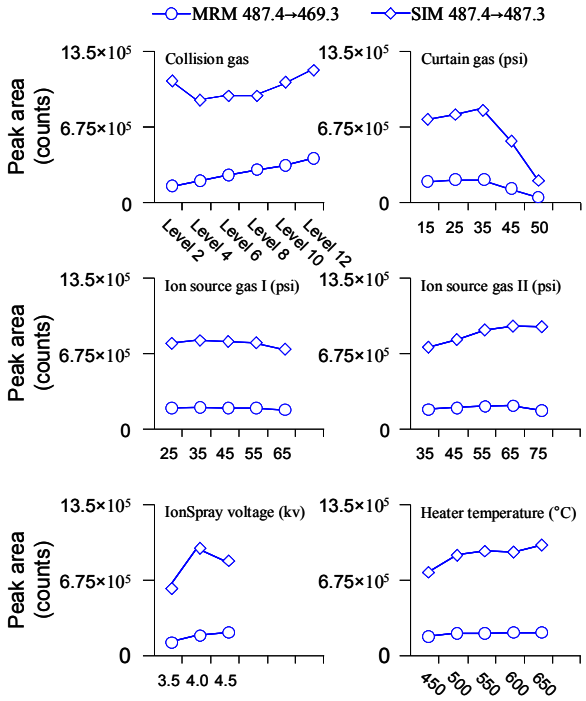


Fig. 2b

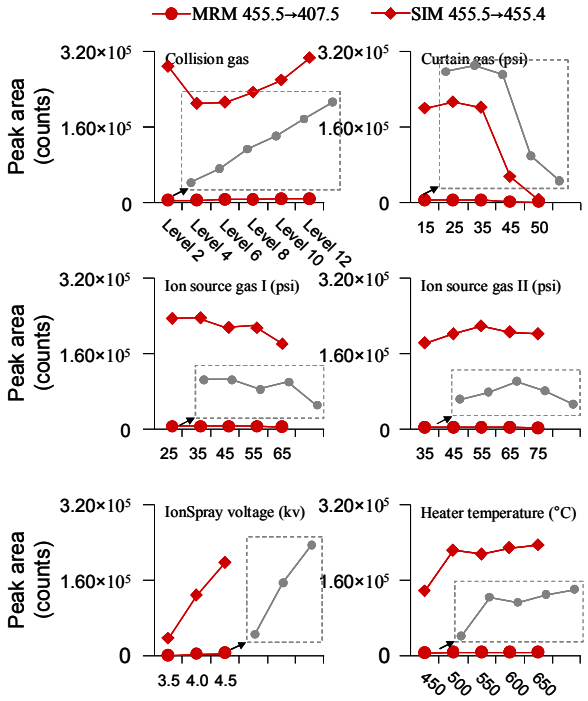


Fig. 3a

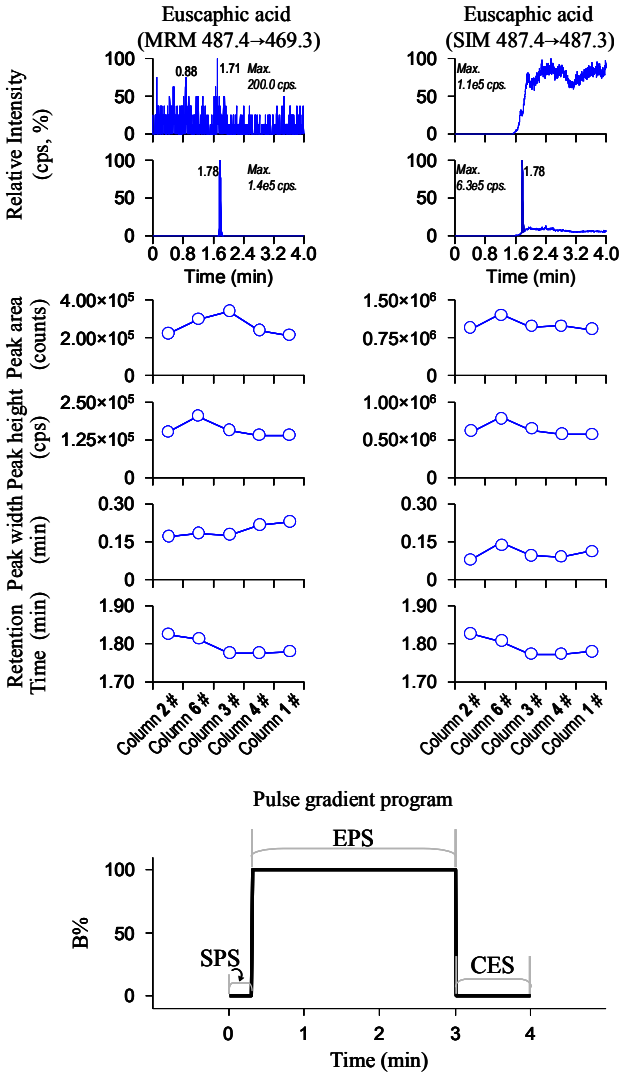


Fig. 3b

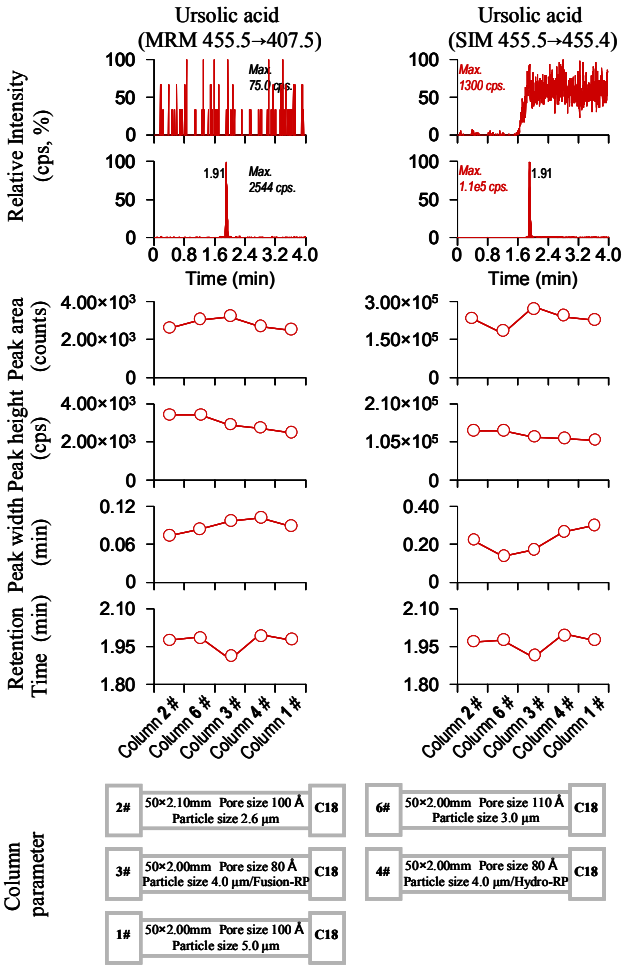


Fig. 4

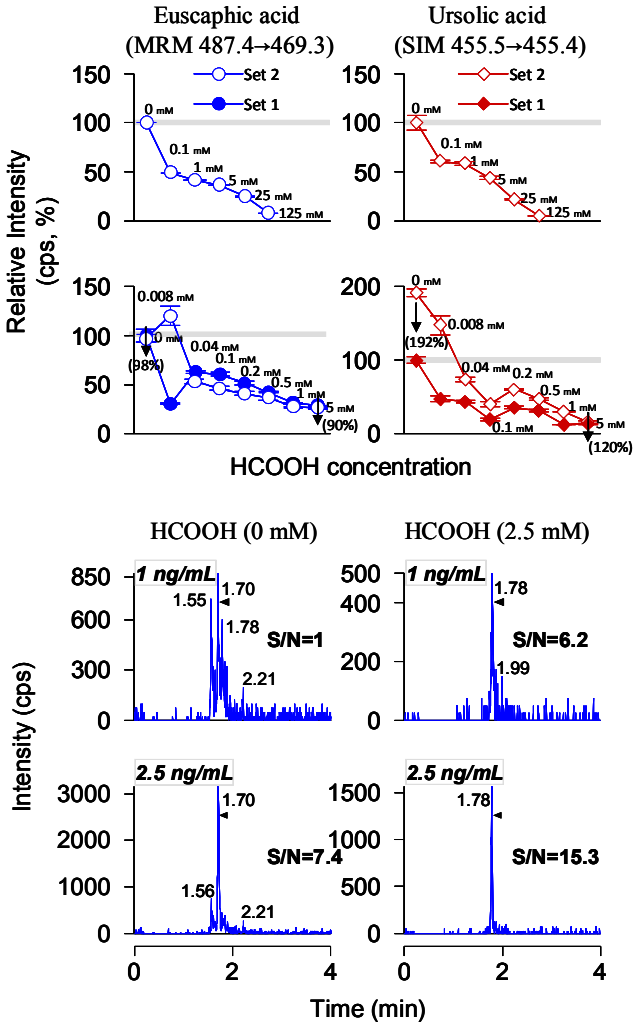


Fig. 5

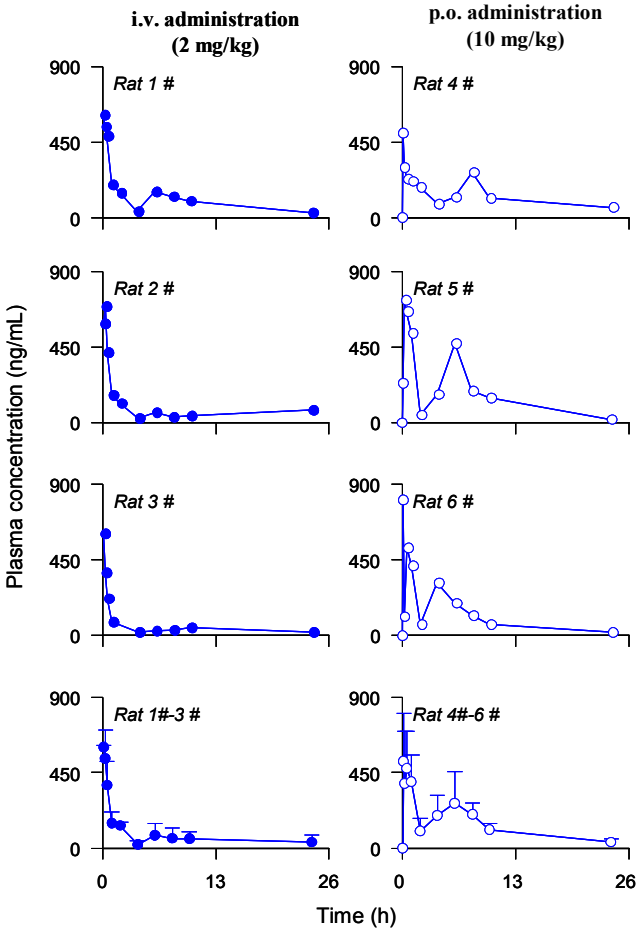


Table 1 Precision and accuracy of the euscaphic acid in rat plasma (n = 6)

Analyte	Spiked concentration (ng/mL)	Intra-day			Inter-day		
		Measured (ng/mL)	RSD (%)	Accuracy (%)	Measured (ng/mL)	RSD (%)	Accuracy (%)
Euscaphic acid	6	5.77 ± 0.51	8.85	96.2 (8.91)	5.88 ± 0.46	7.83	98.1 (7.85)
	60	53.2 ± 2.07	3.88	88.7 (3.92)	54.7 ± 3.02	5.53	91.1 (5.47)
	600	555 ± 30	5.37	92.5 (5.37)	559 ± 32	5.80	93.1 (5.74)

Table 2 Matrix effect and extraction recovery of the euscaphic acid in rat plasma (n = 5)

Analyte	Peak area ($\times 10^3$)									
	Set 1		Set 2		Set 3		Matrix effect		Extraction efficiency	
	Mean ± SD	RSD (%)	Mean ± SD	RSD	Mean ± SD	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Euscaphic acid (ng/mL)										
6	1.41 ± 0.10	7.16	1.41 ± 0.02	1.48	1.27 ± 0.18	14.5	100	1.48	90.4	14.5
60	13.2 ± 0.6	4.38	12.7 ± 0.2	1.63	11.0 ± 0.6	5.10	96.5	1.63	86.4	5.10
600	112 ± 5	4.60	98.1 ± 4.5	4.63	87.5 ± 8.2	9.33	87.9	4.63	89.2	9.30

Table 3 Stability of the euscaphic acid in rat plasma (n = 5).

Spiked	Short-term stability (4h at room temperature)		Autosampler stability (12h at room temperature)		Freeze-thaw stability (3 cycles)	
concentration (ng/mL)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Euscaphic acid						
6	6.00 ± 0.40 (6.66)	100 (6.65)	6.11 ± 0.18 (2.97)	102 (2.99)	5.67 ± 0.31 (5.47)	94.6 (5.48)
60	56.1 ± 3.23 (5.75)	93.5 (5.64)	52.4 ± 2.75 (5.24)	87.3 (5.24)	52.5 ± 0.61 (1.17)	87.4 (1.20)
600	562 ± 36 (6.45)	93.6 (6.36)	538 ± 24 (4.41)	89.8 (4.42)	517 ± 6 (1.23)	86.1 (1.22)

Table 4 Pharmacokinetic data after euscaphic acid administration (2 mg/kg, i.v. and 10 mg/kg, p.o.) to rats (n=3)

PK parameters	i.v. administration (2 mg/kg)	p.o. administration (10 mg/kg)
C_{max} or C_{5min} (ng/mL)	635 ± 52	679 ± 157
T_{max} (h)	0.083	0.083, 0.25
AUC_{0-t} (h·ng/mL)	1521 ± 893	2955 ± 495
$AUC_{0-\infty}$ (h·ng/mL)	1618 ± 1010	3418 ± 636
$t_{1/2}$ (h)	4.61 ± 2.36	-
MRT (h)	8.83 ± 3.30	11.7 ± 5.9
$CL_{tot,\ p}$ (L/h/kg)	1.91 ± 1.68	-
V_{ss} (L/kg)	13.2 ± 5.5	-
F (%)	-	38.9 ± 6.5