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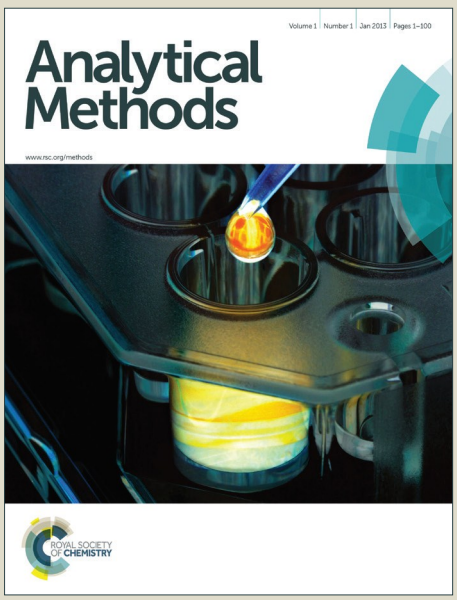

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Determination of α-hederin in rat plasma using liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) and its application to a pharmacokinetic study

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ABSTRACT

The evaluation of efficacy and safety should be paralleled with the assessment of comprehensive pharmacokinetic (PK) properties for a drug candidate and robust bioanalytical method is a prerequisite for obtaining the PK information. α-Hederin is reported to have various *in vitro* and *in vivo* activities; however, very little is known about their PK and metabolic characteristics. In this study, we have developed an efficient LC–ESI(-)–MS/MS assay for α-Hederin and its sapogenin hederagenin in rat plasma. Sample cleanup involved methanol precipitation for identification analysis and liquid-liquid extraction with ethyl acetate for quantification assay. LC analysis was performed under reversed-phase conditions in the modified "pulse gradient elution" mode. Analytes identification and quantification was conducted using multiple reaction monitoring (MRM) mode with euscaphic acid used as internal standard. Under these conditions, deglycosylated metabolites and its sulfate conjugates were measured. But the hederagenin was not detected in rat plasma samples after both oral and intravenous treatments. The mean plasma clearance (CL), volume of distribution (V_{SS}) and elimination half-life ($t_{1/2}$) of α-Hederin was 0.24 $L \cdot h^{-1} \cdot kg^{-1}$, 0.25 $L \cdot kg^{-1}$ and 2.67 h, respectively. The oral bioavailability (*F*) of α-Hederin was about 0.14% in rats, which might result from the poor intestinal absorption and/or extensive biliary excretion. It is hoped that this validated method will be useful for the future PK studies of α-Hederin.

Keywords: α-Hederin, saponin, LC–ESI–MS/MS, bioavailability

Introduction

α-Hederin, an active triterpenoid saponin, shows various biological activities. α-Hederin decreased hepatotoxicity of cadmium in mice through inducing hepatic metallothionein I/II [1] and the mechanism partly involved in upregulation of the metallothionein expression mediated by TNF-α and IL-6 [2]. α-Hederin displayed cytotoxicity towards cancer cell lines [3], strongly inhibited the growth of breast cancer cells and induced apoptosis in these cells via caspase-3 and caspase-9 activation [4]. In addition, α-hederin enhanced 5-fluorouracil cytotoxicity *in vitro* and promoted its antitumor activity when co-prescribed with α-hederin [5]. α-Hederin induced contraction of rat isolated stomach strips [6,7] resulting from the influx of calcium [8] and increased β-adrenoceptor mediated relaxation of airway smooth muscle [9].

It is well known that rational drug discovery needs an early appraisal of pharmacokinetic (PK) properties [10]. Accordingly, the evaluation of efficacy and safety should be paralleled with the assessment of comprehensive PK properties for a drug candidate. For α-hederin, there are no any reports on the PK behaviors in animals or humans. In order to obtain the PK information of this saponin, it is critical to develop robust analytical assays to analyze various biological samples. However, only one study had been reported in this field. Gaillard *et al.* developed a LC–ESI(+)–MS/MS method to detect α-hederin, as well as hederacoside C and hederagenin, in human blood sample collected from an unusual case study [11]. The limit of detection (LOD) for α-hederin was 6 ng·mL^{-1} and this method needed a longer run time (22 min) and a multiple-step biosample preparation procedure. In addition, α-hederin was used as an internal standard (IS) in a LC–ESI(+)–MS/MS method for simultaneous determination of glycyrrhizin and its metabolite glycyrrhetic acid in human plasma [12]. Thus, novel validated methods are needed to facilitate fast and efficient PK evaluation for this compound.

This study aimed to develop and validate a fast LC-MS/MS method to detect the plasma concentration of α-hederin and to apply this method to analyze plasma samples obtained from a single intravenous (i.v.) and oral (p.o.) administration of

α-hederin to Sprague-Dawley (SD) rats. In addition, the in vivo metabolites of α-hederin were also screened. Notably, the deglycosylated metabolite and its sulfate conjugates were detected after i.v. administration of α-hederin to rats. A very low oral bioavailability (*F*, 0.14%) in rats was found.

Experimental

Chemicals and materials

α-Hederin at a purity greater than 98% was separated from the stem of *Hedera nepalensis var. sinensis* (Tobl.) Rehd by associate professor Xiao-Po Zhang in our team. Hederagenin (purity > 98%; MUST-13021002) was purchased from Chengdu MUST Bio-Technology Co., Ltd. (Chengdu, China). Euscaphic acid, used as internal standard (IS), was separated from the root of *Rosa cymosa* and its purity was > 98%. Methanol and acetonitrile of LC grade were products of Tedia Company Inc. (Fairfield, OH, USA). Formic acid (HCOOH) was supplied by Aladdin Industrial Inc. (Shanghai, China). Lithium acetate (CH_3COOLi) was obtained from TCI Development Co., Ltd. (Shanghai, China). Purified water was prepared using the Milipore system (Millipore, Bedford, MA, USA). The other chemical reagents of analytical grade or better were obtained from Hainan YiGao Instrument Co., Ltd (Haikou, China).

LC-MS/MS analysis

The LC-MS/MS apparatus was an AB-SCIEX API 4000 plus mass spectrometer (Toronto, Canada) equipped with a Shimadzu Prominence UFLC chromatographic system (Kyoto, Japan). Chromatography was performed on a Phenomenex Kinetex XB-C_{18} column (2.6 μm, 2.1 mm i.d. × 50 mm) with a temperature stabilized 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 a flow rate of 0.50 $mL \cdot min^{-1}$ consisted of water (0.2‰ HCOOH) for solvent A and acetonitrile (0.2‰ HCOOH) for solvent B. A "pulse gradient" [13] was performed to identify α-hederin and its metabolites with the gradient program as follows: 0–1 min at 1% B; from 1% B to 100% B in 0.01 min (1.01min) and maintained 2 min (1.01–3 min); from 100% B to 0% B in 0.01 min (3.01 min) and maintained 1 min (3.01–4 min). For α-hederin quantification assay, the above-mentioned "pulse gradient" was modified slightly: the elution proportion segment was changed from 70% methanol to 100% methanol

within 2 min.

The mass spectrometer was operated in the negative ESI ion mode with selected multiple reaction monitoring (MRM) mode for α-hederin and IS compound. The spray voltage was set at – 4.5 kV, the heated probe temperature was 600 ºC. The inner coaxial nebulizer N_2 gas (GS1) was 45 psi, the dry N_2 gas (GS2) was 60 psi and the curtain N_2 gas was 25 psi. The in-source collision gas (CAD) flow rate was set at level 12. The MRM of α-hederin and IS (Fig. 1) were m/z 749.5→ 471.4 and 487.4→ 469.3, respectively, with a scan time of 40 ms for each ion pair.

The MRM of α-hederin deglycosylated products, as well as corresponding conjugated metabolites including M_Hed–rhamnose, M_Hed–rhamnose-Glucuronide (+Glu), M_Hed–rhamnose-2Glu, M_Hed–rhamnose-Taurine, M_Hed–rhamnose-Glutamine, M_Hed–rhamnose-Carnitine, M_Hed–rhamnose-Sulfate (+SO_3), M_Hed–rhamnose-2SO_3, M_Hed–rhamnose-Glucosylation, M_Hederagenin-Taurine, M_Hederagenin-Glutamine, M_Hederagenin-SO_3, M_Hederagenin-2SO_3, M_Hederagenin-Glu, M_Hederagenin-2Glu, M_Hederagenin-Glucosylation and oxidated Hederagenin (M_Hederagenin-O) were m/z 603.5→471.4, 779.5→603.5, 955.5→603.5, 710.5→603.5, 732.5→603.5, 747.5→603.5, 683.5→603.5, 763.5→603.5, 765.5→603.5, 578.4→471.4, 600.4→471.4, 551.4→471.4, 631.4→471.4, 647.4→471.4, 823.4→471.4, 633.4→471.4 and 487.4→393.3, respectively, with a scan time of 20 ms for each ion pair.

Calibration and quality control (QC) samples

Appropriate volumes of working solutions were diluted in methanol, where of 10 μL were added to 490 μL of blank plasma then diluted with blank plasma step by step, obtaining seven calibration standards at concentrations from 1 to 2000 ng·mL^{-1} for α-hederin. Low, medium and high concentration QC samples for α-hederin were set at 8, 80 and 800 ng/mL according to a pilot study.

Plasma sample preparation

Methanol precipitation

For identification of α-hederin and its metabolites, the collected plasma samples at different time points (5, 15, 30 min and 1, 2 h, 20 μL each time point) were pooled for each group and the resulting aliquots (100 μL each) were treated with 300 μL methanol and then vortex mixed for 10 min and centrifuged at 18,140*g* for 10 min. The upper supernatant (330 μL) was transferred to a tube and dried under N_2 stream via a Techne^{TM} Sample Concentrator (Bibby Scientific Ltd., Staffordshire, UK). The residue was reconstituted in 50-μL methanol, centrifuged *ditto*, and 10 μL of the resulting supernatant were applied to LC-MS/MS analysis.

Liquid-liquid extraction

For quantification of the α-hederin, the thawed plasma samples (50μL) were extracted with 800 μL of ethyl acetate and centrifuged at 18,140*g* for 10 min. The resulting upper supernatant (720μL) was dried under a stream of N_2 via a Techne^{TM} Sample Concentrator. The residue was reconstituted in 50 μL of methanol containing the IS (500 ng·mL^{-1}), centrifuged *ditto*. Of the clear phase, 10 μL were injected into LC–MS/MS system.

Assay validation

Matrix effects (ME) and extraction efficiencies (EE) were evaluated via a post-extraction spike method [14-16]. Briefly, 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 ME and EE were calculated as follows:

$$ME\ (\%) = (\text{Mean peak area})_{set\ 2} / (\text{Mean peak area})_{set\ 1} \times 100\%$$

$$EE\ (\%) = (\text{Mean peak area})_{set\ 3} / (\text{Mean peak area})_{set\ 2} \times 100\%$$

The other assay validation was implemented according to the U.S. Food and Drug Administration guidance for bioanalytical method validation (www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf).

PK studies

Rat studies were performed in accordance with the Institutional Animal Care and Use

Committee at the Hainan Medical University (Haikou, China). Female Sprague Dawley (SD) rats (200–240 g) were supplied by DongChuang Laboratory Animal Service Department (Changsha, China). The rats were maintained under controlled temperature (24 ± 2°C) and relative humidity (60 ± 10%) with a 12-h light/dark cycle. Rats were acclimated to the facilities and environment for seven days before the experiments. Tap water was available *ad libitum* and the rats were given commercial rat chow *ad libitum* excluding the overnight period before dosing.

For the PK application, α-hederin was dissolved in a mixture containing 6% (v/v) PEG-400, 9.8% (w/v) Tween-80 and 4.4% (v/v) ethanol to achieve a concentration of 1 $mg \cdot mL^{-1}$. Three rats were given a single p.o. dose (10 $mg \cdot kg^{-1}$) of α-hederin and the three more rats were given intravenously (2 $mg \cdot kg^{-1}$). 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 into heparinized tubes. In addition, a PK study of rats receiving vehicle was also implemented for blank control. The blood samples were centrifuged to obtain the plasma fractions which were frozen at –70ºC until analysis.

PK analysis

Plasma PK parameters were calculated by a non-compartmental method using the Kinetica software package (version 3.0; Innaphase Corp., Philadelphia, PA, USA). The maximum concentration in the plasma 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 method. 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

LC-MS/MS conditions optimization

Positive and negative electrospray ionization (ESI) modes were assessed to achieve good specificity and sensitivity for α-hederin and hederagenin measurement. Our results showed that the response in the ESI (-) mode was found to be more sensitive than that of ESI (+) mode by infusing a 1 $\mu g \cdot mL^{-1}$ standard solution of α-hederin in methanol. This is also true for the IS compound [13]. As shown in Fig. 1, the mass spectra for α-hederin, hederagenin and IS reveal peaks at m/z 749.5, 471.3 and 487.4 [13], respectively, as deprotonated molecular ions $[M-H]^-$. The product ion mass spectrum for α-hederin, hederagenin and IS shows the formation of characteristic product ion at m/z 471.4, 393.3 and 469.4, respectively. In addition, the precursor-to-product ion pair of 479.4 ($[M+Li]^+$)→435.4 for α-hederin had higher MS response. However, the selectivity was poor because of the some co-existing interfering substances in the blank plasma sample.

(Insert Fig. 1 here)

In this study, the MRM of α-hederin (749.5→471.4) and hederagenin (471.3→393.3) were selected for further optimization in ESI (-) mode. The MS parameter optimization results are shown in Fig. 2. The peak areas of α-hederin and hederagenin stabilized firstly and then decreased rapidly along with curtain gas value changing from 15 psi to 50 psi. The inflection point value was of 30 psi. Curtain gas flow prevents against ambient air and solvent droplets entering and contaminating the ion optics, while permits direction of sample ions into the vacuum chamber by the electrical fields generated between the vacuum interface and the spray needle. Generally, curtain gas is set as high as possible without losing sensitivity. Therefore, the value (25 psi) before the inflection point was selected for both analytes' quantification. The IonSpray voltage parameter controls the voltage applied to the sprayer, which ionizes the sample in the ion source. This parameter affects the stability of the spray and the sensitivity. As shown in Fig. 2, the peak areas of α-hederin and hederagenin significantly decreased whilst changing from -4.5 kv to

-3.5 kv of IonSpray voltage. Collectively, the optimized ion source parameters for α-hederin and hederagenin were as follows: CAD at level 12, 25 psi for Curtain gas flow, 45 psi for Gas 1, 60 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 than those of data under commonly used MS/MS conditions.

(Insert Fig. 2 here)

Compared with methanol/H_2O system as mobile phase, the acetonitrile/H_2O system had higher peak areas for α-hederin. In this study, we developed a pulse gradient elution method for measurement of α-hederin [13]. We found that the start proportion (SP) of mobile phase B and the start proportion segment (SPS, min) influenced the peak shape and peak response of α-hederin. Finally, 1% B phase for SP and 1 min for SPS were selected.

Method validation

Linearity and lower limit of quantification

The standard curve (Y=0.000402X+0.000811, weight coefficient $1/X$, r=0.9950) was linear over the measured range of 2-2000 ng·mL^{-1} for α-hederin with correlation coefficient of 0.994. The lower limit of quantification (LLOQ) was 2 ng·mL^{-1} for this analyte. A representative chromatogram is showed in Fig. 3. No peaks from endogenous biological matrix or other sources were observed at the same retention time of α-hederin and IS in any of the blank plasma, which suggested that the developed method was specific and selective.

Accuracy and precision of the assay

Within- and between-day precision and accuracy data are summarized in Table 1. Accuracy, ranging from 88.5% to 107%, was well in line with the U.S. FDA guidance. Within- and between-day deviations were always less than 12.8% for α-hederin, which were within the acceptable criteria.

(Insert Table 1 and Fig. 3 here)

Matrix effects (ME) and extraction efficiencies (EE)

As shown in Table 2, for α-hederin, the EE ranged from 77.8% to 95.9% with relative standard deviations (RSDs) less than 13.3%. The average ME at all measured concentrations were 87.8–96.5% and the RSDs were no more than 6.84%. The EE and ME of both the α-hederin and IS compound were all within the acceptable range.

(Insert Table 2 here)

In this assay, we found that the peak responses of α-hederin significantly declined after multiple injections using the above-mentioned "pulse gradient elution". The elution proportion (EP, B%) and elution proportion segment (EPS, min) influenced the elution of potential interfering substances in the rat plasma matrix. The EP was adjusted from 100% B to 70% B and the EPS set as 70% B to 100% B within 2 min. Based on these modifications for LC conditions, the matrix interfering was overcome. In addition, the HCOOH concentration in the mobile phase also affected the ME. Previously, we have found that low concentration level of HCOOH (0.1‰ and 0.2‰) [13] was suitable for euscaphic acid, *i.e.*, IS compound in this study. For α-hederin, the inclusion of 0.2‰ HCOOH into the mobile phase enhanced its signal intensity.

Stability

Drug stability in the rat plasma is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. The stability evaluations results are summarized in Table 3.The storage of plasma samples at room temperature for 4 h (pre-treatment) alter signal responses of α-hederin. Processed samples (post-treatment) were stable at auto-sampler room for 8 h. The overall accuracy between initial and final analysis were between 88.0% and 114%, with RSD always less than 10.0%. Therefore, α-hederin was stable under the tested conditions.

(Insert Table 3 here)

PK study

Deglycosylated metabolites and phase II metabolites of α-hederin in rat plasma

Saponins could be stripped of their sugar moieties by the colonic microflora [17,18].

In this study, we proposed that this molecule might be transformed to deglycosylated metabolites including its sapogenin, *i.e.*, hederagenin. Moreover, one carboxyl group (-COOH) and one hydroxyl group (-OH) exist in the hederagenin, which might be coupled to endogenous conjugating substances (such as glucuronic acid) producing glucuronides, sulfates and other metabolites [19-21]. Figure 4 depicts the deglycosylated metabolites and phase II metabolites in rat plasma after administration of α-hederin to rats. After a single i.v. medication, deglycosylated metabolite (stripped of rhamnose moiety, t_R=2.31 min) and its sulfate product (t_R=2.28 min) were measured. Hederagenin, stripped of rhamnose-arabinose moiety, could not be detected. However, its sulfate conjugate was found in the rat plasma samples. After p.o. ingestion of α-hederin, only hederagenin sulfate could be obviously measured. For M_α-hederin-rhamnose+SO_3, this metabolite needed further identification because of its poor peak area response. A previous report showed that the mean C_{max} of hederagenin was about 48 ng·mL^{-1} after orally administered a mixture containing hederagenin (280 mg·kg^{-1}) to rats [22]. Therefore, the systemic exposure level was relatively low.

(Insert Fig. 4 here)

Rat plasma PK parameters of α-hederin

The newly validated method was used to quantify plasma concentration of α-hederin after a single p.o. and i.v. administration of α-hederin to SD rats. The plasma concentration-time curves of α-hederin after medication are shown in Fig. 5. The key PK parameters are summarized in Table 4.

(Insert Fig. 5 and Table 4 here)

Plasma α-hederin was measured up to 10 h after i.v. dosing (Fig. 5), with the mean maximum plasma concentration of 10.5 μg·mL^{-1} (14 μM). The mean plasma $t_{1/2}$ value was 2.67 h. The mean $CL_{tot,p}$ value was 0.24 L·h^{-1}·kg^{-1}. The V_{SS} was 0.25 L·kg^{-1} and less than the rat total body water by volume (0.67 L·kg^{-1}) [23], suggesting that this molecule might tend to be restricted to the bloodstream and did not enter the tissues in

significant amounts. For oral administration, α-hederin was monitored in all the rat plasma samples only up to 6-8 h and the concentrations of the 10- and 24-h samples were less than LLOQ. The plasma concentration-time curves displayed the mean maximum concentrations of 14.5 ng·mL^{-1} (0.02 μM) and the mean T_{max} was 1.17 h. The mean oral *F* of α-hederin was less than 1% (0.14%). Some other saponins also had very low oral *F* in rats such as ginsenoside Ra3 and Rd (0.1-0.2%), ginsenoside Re (0.2-0.6%) and dioscin (0.2%) [24]. Poor intestinal absorption and extensive biliary excretion might contribute to limit the oral *F* of this molecule. We still have a poor understanding of the modes of action and relative efficacy of α-hederin under such a lower oral *F* compared with synthetic drugs. α-Hederin is always administered orally and inevitably exposed to the gut microbiota, so this saponin might work both by modulating gut microbiota to regain ecological balance and by regulating genes within the host to regain metabolic/immune homeostasis [25].

Conclusion

In summary, we developed and validated a rapid LC–ESI (-)–MS/MS method for measurement of α-hederin in rat plasma and successfully applied this method to a rat PK study. MS conditions and LC conditions were optimized. Notably, the systemic exposure level of hederagenin was very low. The deglycosylated metabolite and its sulfate conjugate, as well as hederagenin sulfate metabolite, were detected after i.v. administration of α-hederin to rats. A very low oral *F* (0.14%) in rats was found might resulting from poor intestinal absorption and/or extensive biliary excretion. The microbiotal degalycosylation and the subsequent sapogenin metabolism, as well as route of elimination, are warranted in the future.

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Competing interests

There are no competing interests to declare.

References

[1] J. Liu, S. Choudhuri, Y. Liu, H. Kreppel, G.K. Andrews, C.D. Klaassen, *Toxicol. Appl. Pharmacol*., 1993, **121**, 144-151.

[2] J.Y. Kim, C.Y. Choi, H.G. Jeong, *Planta Med.*, 2005, **71**, 743-747.

[3] V. Kuete, S.B. Tankeo, M.E. Saeed, B. Wiench, P. Tane, T. Efferth, J. Ethnopharmacol., 2014, **153**, 207-219.

[4] L. Cheng, T.S. Xia, Y.F. Wang, W. Zhou, X.Q. Liang, J.Q. Xue, L. Shi, Y. Wang, Q. Ding, M. Wang, *Int. J. Oncol.*, 2014, **45**, 757-763.

[5] S.S. Bun, R. Elias, B. Baghdikian, J. Ciccolini, E. Ollivier, G. Balansard, *Phytother. Res.*, 2008, **22**, 1299-1302

[6] M. Mendel, M. Chłopecka, N. Dziekan, W. Karlik, M. Wiechetek, *Phytomedicine*, 2012, **19**, 591-595.

[7] M. Mendel, M. Chłopecka, N. Dziekan, M. Wiechetek, *J. Ethnopharmacol.*, 2011, 134, 796-802.

[8] M. Mendel, M. Chłopecka, N. Dziekan, W. Karlik, M. Wiechetek, *J Ethnopharmacol.*, 2013, **146**, 423-426.

[9] A. Wolf, R. Gosens, H. Meurs, H. Häberlein, *Phytomedicine*, 2011, **18**, 214-218.

[10] I. Kola, J. Landis, *Nat. Rev. Drug Discov.*, 2004, **3**, 711-715.

[11] Y. Gaillard, P. Blaise, A. Darré, T. Barbier, G. Pépin, *J. Anal. Toxicol.*, 2003, **27**, 257-262.

[12] Z.J. Lin, S.X. Qiu, A. Wufuer, L. Shum, *J. Chromatogr. B.*, 2005, **814**, 201-207.

[13] F. Chen, H.L. Li, Y.F. Tan, W.Y. Lai, Z.M. Qin, H.D. Cai, Y.H. Li, J.Q. Zhang, X.P. Zhang, *Anal. Methods*, 2014, **6**, 8713-8721.

[14] L. Li, S. Liang, F. Du, C. Li, *J. Am. Soc. Mass Spectrom.*, 2007, **18**, 778-782.

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

[16] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, *Anal. Chem.*, 2003, **75**, 3019-3030.

[17] H. Liu, J. Yang, F. Du, X. Gao, X. Ma, Y. Huang, F. Xu, W. Niu, F. Wang, Y. Mao, Y. Sun, T. Lu, C. Liu, B. Zhang, C. Li, *Drug Metab. Dispos.*, 2009, **37**, 2290-2298.

[18] Z. Hu, J. Yang, C. Cheng, Y. Huang, F. Du, F. Wang, W. Niu, F. Xu, R. Jiang, X. Gao, C. Li, *Drug Metab Dispos.*, 2013, **41**, 1457-1469.

[19] F. Chen, L. Li, F. Xu, Y. Sun, F. Du, X. Ma, C. Zhong, X. Li, F. Wang, N. Zhang, C. Li, *Br. J. Pharmacol.*, 2013, **170**, 440-457.

[20] 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.

[21] M. Holcapek, L. Kolárová, M. Nobilis, *Anal. Bioanal. Chem.*, 2008, **391**, 59-78.

[22] X. Yang, G. Li, L. Chen, C. Zhang, X. Wan, J. Xu, *J. Chromatogr. B.*, 2011, **879**, 1973-1979.

[23] B. Davies, T. Morris, *Pharm. Res.*, 1993, **10**, 1093-1095.

[24] K. Yu, F. Chen, C. Li, *Curr. Drug Metab.*, 2012, **13**, 577-598.

[25] L. Zhao, J.K. Nicholson, A. Lu, Z. Wang, H. Tang, E. Holmes, J. Shen, X. Zhang, J.V. Li, J.C. Lindon, *J. Proteome. Res.*, 2012, **11**, 3509-3519.

Fig. 1 Chemical structures and MS/MS spectra of α-hederin, hederagenin and euscaphic acid (IS).

Fig. 2 MS parameters optimization for α-hederin and hederagenin. 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 LC-MS/MS chromatograms for a typical blank rat plasma sample (panel A), the same blank plasma sample spiked with euscaphic acid (IS, panel B), a mixture of standard α-hederin and IS (panel C), and an IS-spiked plasma sample obtained from a rat 5 min after receiving a single oral dose of α-hederin at 10 $mg \cdot kg^{-1}$ (panel D).

Fig. 4 Representative LC-MS/MS chromatograms for identification of deglycosylated metabolites and its sulfated metabolites of α-hederin in rat plasma after a single p.o. dose (10 $mg \cdot kg^{-1}$) and an i.v. dose (2 $mg \cdot kg^{-1}$) of α-hederin (panel A). Proposed fragmentation pathways of α-hederin (panel B).

Fig. 5 Plasma concentration-time profiles of α-hederin after a single i.v. (2 $mg \cdot kg^{-1}$, left panel) and p.o. (10 $mg \cdot kg^{-1}$, right panel) administration to rats.

Fig. 1

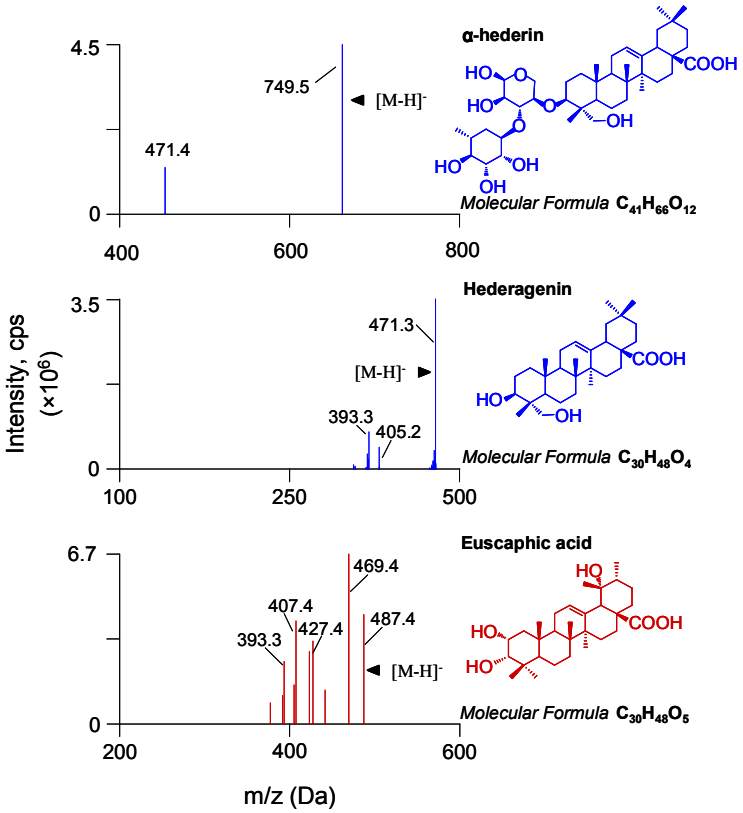

Fig. 2

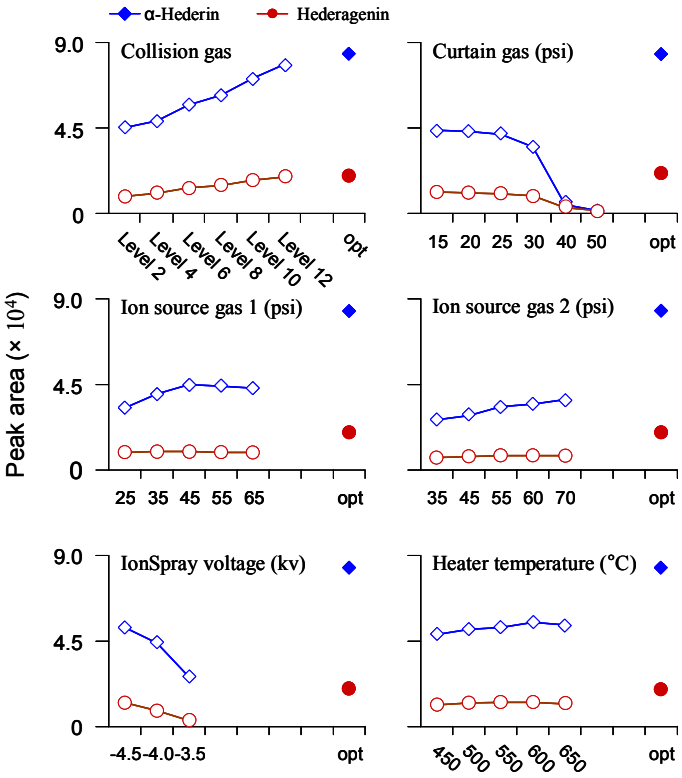

Fig. 3

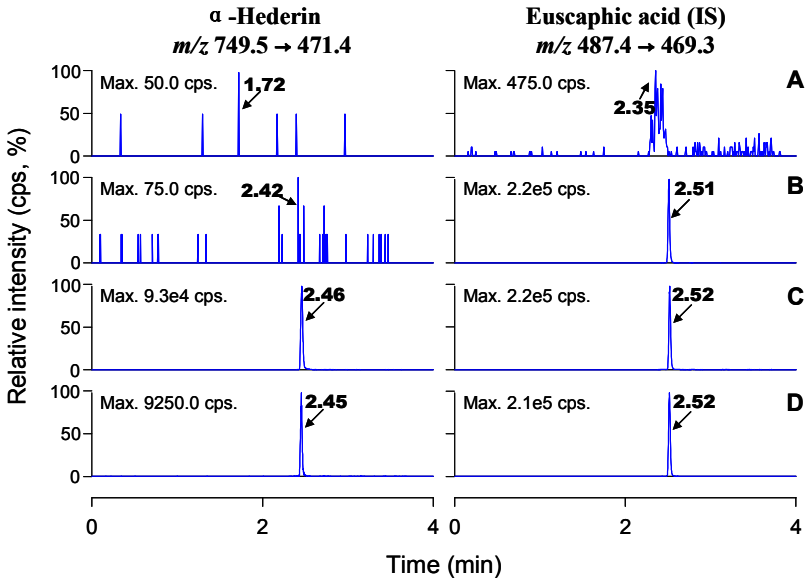

Fig. 4

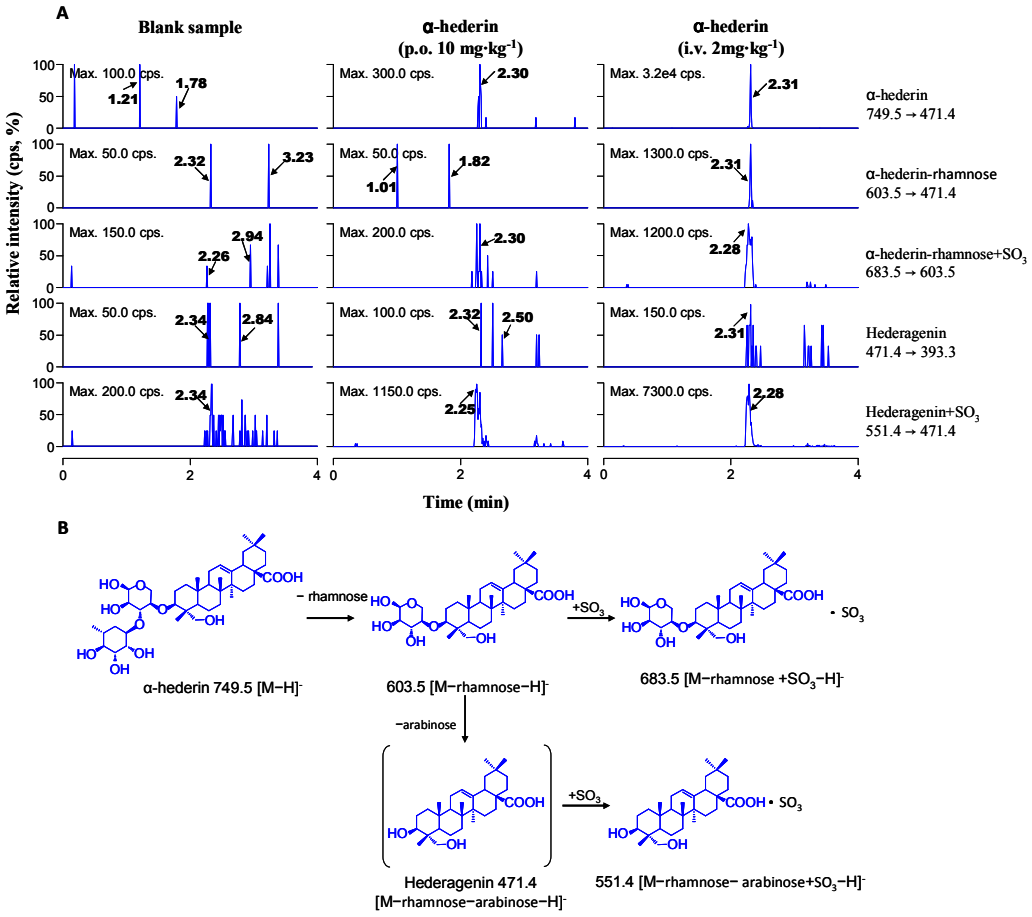

Fig. 5

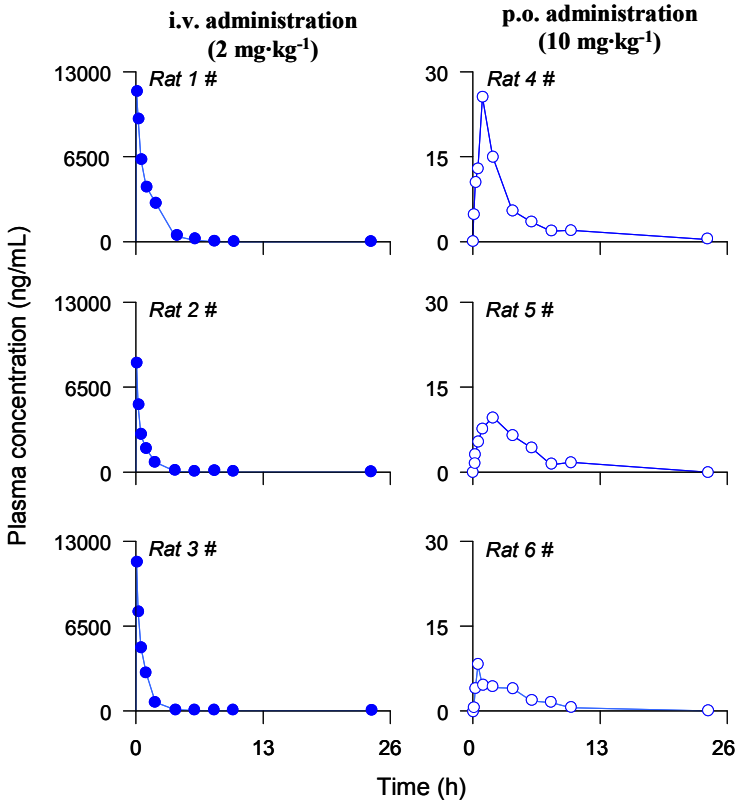

Table 1 Precision and accuracy of α-hederin in rat plasma (n = 6)

Analyte	Spiked concentration (ng/mL)	Within-day			Between-day		
		Measured (ng/mL)	RSD (%)	Accuracy (%)	Measured (ng/mL)	RSD (%)	Accuracy (%)
α-hederin	8	7.65 ± 0.98	12.8	95.7 (13.0)	7.62 ± 0.79	10.3	95.2 (10.4)
	80	70.8 ± 7.38	10.4	88.5 (10.4)	71.6 ± 6.99	9.77	89.5 (9.72)
	800	849 ± 75.9	8.94	106 (8.90)	854 ± 66.4	7.78	107 (7.73)

Table 2 Matrix effect and extraction recovery of α-hederin and IS 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 (%)
α-hederin (ng/mL)										
8	1.95 ± 0.14	7.22	1.88 ± 0.10	5.07	1.46 ± 0.07	4.62	96.5	6.84	77.8	7.16
80	16.9 ± 1.51	8.94	15.4 ± 1.55	10.1	12.7 ± 1.18	9.32	90.9	6.64	82.3	13.3
800	128 ± 2.51	1.97	112 ± 2	1.79	107 ± 3.65	3.40	87.8	2.71	95.9	2.50
IS (ng/mL)										
500	311 ± 36.8	11.9	304 ± 24.3	8.00	215 ± 20.0	10.8	97.7	7.07	70.7	14.2

Table 3 Stability of the α-hederin in rat plasma (n = 5).

Spiked concentration (ng/mL)	Short-term stability (4h at room temperature)		Autosampler stability (8h at room temperature)		Freeze-thaw stability (3 cycles)	
	Mean ± SD	Accuracy (%)	Mean ± SD	Accuracy (%)	Mean ± SD	Accuracy (%)
α-hederin						
8	8.00 ± 0.63 (7.89)	100 (7.83)	8.46 ± 0.60 (7.08)	106 (7.12)	7.25 ± 0.73 (10.1)	90.6 (10.0)
80	70.4 ± 6.01 (8.54)	88.0 (8.56)	77.0 ± 4.25 (5.53)	96.3 (5.68)	80.1 ± 5.07 (6.33)	100 (6.32)
800	907± 52.9 (5.83)	113 (5.84)	913 ± 65.6 (7.18)	114 (7.02)	854 ± 41.4 (4.84)	107 (4.88)

Table 4 PK parameters of α-hederin after dosing (n=3)

PK parameters	i.v. administration (2 mg/kg)	p.o. administration (10 mg/kg)
C_{max} or C_{5min} ($ng \cdot mL^{-1}$)	10460 + 1804	14.5 ± 9.6
T_{max} (h)	0.083	1.17 ± 0.76
AUC_{0-t} ($h \cdot ng \cdot mL^{-1}$)	9391 ± 4293	50.7 ± 21.0
$AUC_{0-\infty}$ ($h \cdot ng \cdot mL^{-1}$)	9392 ± 4292	55.1 ± 21.6
$t_{1/2}$ (h)	2.67 ± 0.56	-
MRT (h)	1.07 ± 0.28	4.94 ± 1.51
$CL_{tot, p}$ ($L \cdot h^{-1} \cdot kg^{-1}$)	0.24 ± 0.10	-
V_{ss} ($L \cdot kg^{-1}$)	0.25 ± 0.09	-
F (%)	-	0.14 ± 0.02%