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HPLC-ESI-MS/MS Validation and Pharmacokinetics of Kalopanaxsaponin A in Rats

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ABSTRACT

Kalopanaxsaponin A (KPS-A) is a potential anti-tumor active compound isolated from the stems of *Stauntonia obovatifoliola* Hayata subsp. *intermedia*. A rapid and accurate high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was firstly developed and validated for the quantification of KPS-A in rat plasma. The plasma samples were pretreated by a simple protein precipitation (PPT) procedure with methanol: acetonitrile (1:1, v/v). Chromatographic separation was successfully accomplished on an Agilent Zorbax XDB C_{18} column (2.1 mm × 50 mm, 3.5 μm) with a gradient elution system composed of 0.1% formic acid aqueous solution and 0.1% formic acid in acetonitrile solution. The flow rate was set to be 0.50 mL min^{-1}. The multiple reaction monitoring (MRM) was based on the transitions of *m/z* = 749.4→585.5 for KPS-A and 268.9→158.8 for genistein (IS). The assay was validated to demonstrate the selectivity, linearity, recovery, accuracy, precision and stability. The lower limit of quantification (LLOQ) was 0.50 ng mL^{-1} in 25 μL of rat plasma. The developed and validated method has been successfully applied to the quantification and pharmacokinetic study of KPS-A in rats after intravenous and oral administration of KPS-A. The oral absolute bioavailability (F) of KPS-A was calculated to be 0.006 ± 0.002%, suggesting its very poor absorption and/or strong metabolism *in vivo*.

Key Words: *Stauntonia obovatifoliola* Hayata subsp. *intermedia*; Kalopanaxsaponin A; Pharmacokinetics; LC-ESI-MS/MS; Rat

1. Introduction

Stauntonia obovatifoliola Hayata subsp. *intermedia* (Wuzhinateng in Chinese), which belongs to the genus *Stauntonia* (family *Lardizabalaceae*), is a wild plant distributed in the southern regions of China [1]. Its stem is a classical herbal drug in the traditional Yao medicine of Chinese ethnomedicine [2], which traditionally treat rheumatic arthralgia, headache, visceral pain, neuralgia, heat strangury, hernia pain and trauma pain [3].

Kalopanaxsaponin A (KPS-A, chemical structure shown in Fig.1) was one kind of oleanane-type triterpenoid saponins isolated and identified from *S. obovatifoliola* Hayata subsp. *intermedia* and other natural plants [4-8]. Extensive phytochemical and pharmacological studies have confirmed that KPS-A exhibits significant anti-inflammatory [9-12], anti-lipid peroxidative [13, 14], vasoactive [15], anti-nociceptive and anti-inflammatory effects [16-18]. Furthermore, KPS-A inhibits the invasion of human oral squamous cell carcinoma by reducing metalloproteinase-9 mRNA stability and protein trafficking [19]. KPS-A also shows strong inhibition effects on several tumor cells [20-24]. As a result, KPS-A is now being a novel drug candidate targeted to several tumors in our laboratory.

Based on the relative large molecule weight of KPS-A, its bioavailability might be of great concern in the period of early discovery. Therefore, it is necessary to develop a rapid and accurate bioanalytical method to quantify KPS-A in biological fluids and then apply to the bioavailability study of KPS-A in animals. However, there is no reported bioanalytical method for the quantification of KPS-A in biological fluids, even for the quantifications of other structure similar compounds, such as Kalopanaxsaponin B, Kalopanaxsaponin J, Kalopanaxsaponin K and Kalopanaxsaponin H. These compounds show the same skeleton structure as KPS-A, but they have different carbohydrate side chains [25]. Hence the pharmacokinetic profile of KPS-A has not been investigated.

Thus, the aim of this present work was to develop a rapid and accurate bioanalytical method for the quantification of KPS-A in rat plasma. The developed method was further validated and then applied to a pharmacokinetic study of KPS-A in rats after intravenous and oral administration of KPS-A.

2. Experimental

2.1 Chemicals and reagents

Reference standard of kalopanaxsaponin A (KPS-A, HPLC purity ≥ 98%, chemical structure shown in Fig.1) was isolated from the stems of *S. obovatifoliola* Hayata subsp. *intermedia* in our laboratory [25]. The separation, purification, identification and quantification of KPS-A were performed as follows: dry stems of *S. obovatifoliola* Hayata subsp. *intermedia* were smashed by a pulveriser (through 50-mesh) and refluxed with 60% v/v ethanol. The extracts were concentrated to dryness and then suspended in water and extracted sequentially with ethyl acetate and butyl alcohol saturated with water. The butyl alcohol-soluble fraction was subjected to column chromatography on D_{101} macroporous resin (Tianjin Haiguang Chemical Co., Ltd., Tianjin, China) and successively eluted by 10%, 30%, 50% and 70 % v/v ethanol. The 70% v/v ethanol fraction was subjected on silica gel (200-300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) using an elution system of dichloromethane and methanol (20:1~10:1~4:1) to yield the crude crystal of KPS-A. Then it was purified on an octadecylsilanised (ODS) silica gel column (Merck, Darmstadt, Germany). Finally, the purity of KPS-A was further evaluated to be greater than 98.0% by HPLC–DAD method at UV 210 nm using area normalization procedure. KPS-A was obtained as white amorphous powders, which gave positive results for the *Liebermann-Burchard* reaction and *Molish* reagent. The molecular formula was determined based on the associated HR-ESI-MS spectra. Their respective ^{1}H NMR and ^{13}C NMR spectral results were compared against data reported in the literature [26], see supplementary materials.

Reference standard of genistein (Internal standard, IS, batch No. 20130901, chemical structure shown in Fig.1) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Polyethylene glycol 400 (PEG 400, batch No. 10150733) was purchased from Alfa Aesar (Heysham, Lancs, UK). Propylene glycol (PG, batch No. CO3SE10U04) and sodium carboxyl methyl cellulose (CMC-Na, batch No. 20130307) were both purchased from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd (Beijing, China). Dimethyl sulfoxide (DMSO, batch No. 2543C551) was purchased from Amresco Inc. (Solon, OH, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other chemicals were of analytical grade.

2.2 Preparation of standard and quality control solutions

KPS-A reference standard was accurately weighed and dissolved in dimethyl sulfoxide (DMSO), and then diluted to appropriate concentrations using methanol for establishment of calibration curves in rat plasma. The concentration of stock solution of KPS-A was 1000 μg mL^{-1}. Working solutions of KPS-A were prepared at concentrations of 5.0, 10, 20, 50, 200, 500, 2000 and 5000 ng mL^{-1}. IS (Genistein) working solution (400 ng mL^{-1}) was prepared by diluting with methanol:acetonitrile (50:50, v/v). All the stock and working standard solutions were stored at 4°C prior to use.

2.3 Sample preparation

After thawed at room temperature for about 30 min and vortexed for 30 s, aliquots of 25 μL rat plasma were mixed with 2.5 μL of methanol (or standard or QC solution) and 100 μL of IS solution (400 ng mL^{-1} genistein in methanol:acetonitrile (50:50, v/v)). After vortexed for 1 min and then centrifuged at 12000 *g* for 10 min, aliquots of 75 μL supernatants were transferred to HPLC vials. A volume of 10 μL of this solution was then injected onto the column.

2.4 Instrumentation and analytical conditions

The HPLC system equipped with an LC-20AD pump, a DGU-20 A_3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Kyoto, Japan) was used in the study. Chromatographic separation was performed on an Agilent Zorbax XDB C_{18} column (2.1 mm × 50 mm, 3.5 μm) with a gradient elution by a mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) with following gradient: 0.00 min 10% B, 0.30 min 10% B, 1.00 min 80% B, 2.50 min 95% B, 2.51 min 10% B, 3.50 min 10% B, with the flow rate of 0.50 mL min^{-1}. The injection volume was set to be 10 μL.

An API 4000 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface was coupled with HPLC system. The ESI source was operated in negative mode and the curtain, nebulizer and turbo-gases (all nitrogen) were set at 15, 65 and 55 psi respectively, following optimization of the setting parameters. The source temperature was set to be 550ºC and the ion spray needle voltage was -4200 V. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3 in the multiple reaction monitoring (MRM) mode, with a dwell time of 150 ms per multiple reaction monitoring channel. The main MS parameters of KPS-A and genistein (IS) were shown in Table 1. The Analyst Data Acquisition and Processing software (Version 1.5.2, Applied Biosystems/MDS Sciex, Concord, ON, Canada) was used to collect and analyze the data.

2.5 Calibration curves

Calibration standards were prepared freshly on the day of analysis, in duplicate, by addition of 2.5 μL aliquots of appropriate working solutions and 100 μL of IS solution (400 ng mL^{-1} genistein in methanol: acetonitrile (50:50, v/v)) to 25 μL aliquots of blank rat plasma (drug-free) at the following concentrations: 0.50, 1.00, 2.00, 5.00, 20.0, 50.0, 200, and 500 ng mL^{-1}. Six pools of each QC sample were prepared in

blank rat plasma at concentrations of 1.00 ng mL^{-1} (Low), 20.0 ng mL^{-1} (Medium) and 400 ng mL^{-1} (High). All the solutions were stored at -20°C prior to use.

2.6 Method validation

According to the international guidelines [27, 28], the method was validated in terms of the selectivity, matrix effect, linearity, lower limit of quantification (LLOQ), accuracy, precision, recovery and stability.

The selectivity was evaluated by analyzing six individual samples of blank rat plasma by comparison with the plasma-spiked analyte for endogenous interferences. Each blank sample was tested by LC-MS/MS method in order to investigate potential interferences.

The matrix effect was determined by examining the ratio of the peak areas of KPS-A dissolved in blank matrix extract to that in standard solution containing equivalent amounts of the compounds. The procedure was repeated five times. The matrix effect of IS was determined in the same way.

The linearity of the method was determined by analyzing a series of standard plasma samples at concentrations of 0.50, 1.00, 2.00, 5.00, 20.0, 50.0, 200, and 500 ng mL^{-1} for KPS-A by least squares linear regression of the peak area ratios of KPS-A to IS obtained against the corresponding concentration (x) with a weighting factor of $1/x^2$. The LLOQ was defined as the lowest concentration on the calibration curve with acceptable precision and accuracy (<15%). The criteria for the calibration included a correlation coefficient (r) of 0.995 or better. Concentrations in the QCs and unknown plasma samples were quantified by using the internal standard calibration method.

The precision and accuracy of method were obtained by performing replicate analyses of QC samples at three concentrations in five replicates. Each concentration level were evaluated on the same day for intra-day precision, while repeated analysis at each concentration of QC samples five times per day over five consecutive days for inter-day precision and accuracy. Standard deviations (SD) and relative

standard deviations (RSD, %) were calculated from the QC values and used to estimate the inter- and intra-day precision.

Recoveries of KPS-A and IS from rat plasma were determined by comparing of the responses of KPS-A and IS in plasma carried through the complete preparation procedure to those spiked into the prepared blank plasma of the same concentration as those of QC samples, respectively.

The stability of KPS-A in ethanol solution has been investigated in our laboratory [25]. It was found that KPS-A was stable in ethanol solution when stored at 4 °C. Therefore, the stability of KPS-A in rat plasma was further assessed by analyzing QC samples at three concentrations exposed to different time and temperature conditions. The long-term stability was assessed after the QC samples had been stored at -80°C for 1 month. The freeze-thaw stability was determined after three freeze-thaw cycles (-20°C to 20°C) on 3 consecutive days. The amount of KPS-A in plasma samples was determined using a newly prepared calibration curve. Stability of KPS-A was expressed as a percentage of nominal concentration. Deviation of the stability results should be within ±15% of the nominal values.

2.7 Application to a pharmacokinetic study

Six male Sprague-Dawley rats (weighing 220 ± 30 g, 8 weeks) were purchased from Beijing Vital River Laboratories Co., Ltd (Beijing, China). All experimental procedures were approved by the Experimental Animal Care and Use Committee of Capital Medical University (Beijing, China). The animals were housed in an air-conditioned room at a temperature of 23 ± 2°C, with a relative humidity of 55 ± 10%, an illumination intensity of 150 - 300 lx, a frequency of air ventilation of 15 - 20 times hr^{-1} and a 12 hr illumination. Food and water were supplied *ad libitum*.

Two days before the experiment, polyethylene cannulas were inserted in the right external jugular vein of the rats while they were anesthetized with pentobarbital (50 mg kg^{-1}, intravenous administration). To

prevent blood clotting, the cannulas were externalized at the back of the neck and filled with heparinized saline (20 units mL^{-1}). The rats from oral group were fasted for 16 hr before experiments with the exception of free access to water, while the rats from intravenous group were free access to standard diet and water. The intravenous dosing solution with KPS-A concentration of 2.0 mg mL^{-1} was prepared by dissolving appropriate amount of KPS-A in DMSO:PEG400:PG:H_2O (5:30:30:35, v/v/v/v) and filtering through 0.22 μm Millipore filter prior to use. The oral dosing suspension with KPS-A concentration of 20 mg mL^{-1} was prepared by dissolving appropriate amount of KPS-A in 0.5% CMC-Na solution. The intravenous and oral doses of KPS-A were 5 mg kg^{-1} and 100 mg kg^{-1}, and the intravenous and oral dose volumes were 2.5 mL kg^{-1} and 5.0 mL kg^{-1}, respectively. After intravenous administration of 5 mg kg^{-1} KPS-A through tail vein, aliquots of 0.10 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.033, 0.083, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 12, 24, 36 and 48 hr). After oral administration, aliquots of 0.10 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.083, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 12, 24, 36 and 48 hr). Heparinized blood was centrifuged at 12000 *g* at room temperature for 5 minutes to obtain plasma, which was stored at -80°C until analysis.

DAS Version 2.0 (Chinese Pharmacological Society, Beijing, China) were employed to analyze pharmacokinetic parameters including half-life ($t_{1/2}$), maximum plasma time (t_{max}) and concentration (C_{max}), area under concentration–time curve (AUC_{0-t} and $AUC_{0-\infty}$), clearance (CL), steady-state volume of distribution (V_Z), mean residence time (MRT) of EG were analyzed by non-compartmental method. All results were expressed as arithmetic mean ± standard deviation (SD).

3. Results and discussion

3.1 Mass spectrometry and chromatography

KPS-A is one kind of oleanane-type triterpenoid saponin, and thus shows only very weak end absorption in the ultraviolet zone. Due to its poor absorption, extensive metabolism and strong first-pass effects, it is predicted that the concentrations of KPS-A in rat plasma will be very low after oral administration (~ng mL^{-1}). As a result, the sensitive and selective LC-MS/MS method is our first choice in the period of method development.

The present study firstly describes the development of a rapid, sensitive and selective LC-MS/MS assay for the determination of KPS-A concentrations in rat plasma. The full-scan product ion mass spectra of KPS-A and genistein are shown in Fig.1. In the full-scan Q1 mass spectrum, the parent negative ion peak of KPS-A appeared at *m/z* = 749.4, and the abundance of this ion peak was sufficient for the quantification of KPS-A. Therefore, *m/z* 749.4 $[M-H]^-$ was finally utilized to the parent ion in present study. For KPS-A, several product ions with similar abundances presented in the full-scan product ion mass spectra of KPS-A, including *m/z* 585.5 ($[M-H-C_6H_{11}O_5]^-$), 471.3 ($[M-H-C_{11}H_{18}O_8]^-$), and 423.3 ($[M-H-H_2-C_{11}H_{19}O_8-CO_2]^-$), in which product ion *m/z* 585.5 was most stable and abundant, but not *m/z* 471.3, even though the response of *m/z* 471.3 seemed to be the highest. In order to avoid false positive results, two other characteristic product ions *m/z* 471.3 and *m/z* 423.3 had been chosen to be the product ions for confirmation (see Table 1). For genistein, the most abundant peak was the protonated molecular ion $[M-H]^-$ found at *m/z* = 268.8. Thus, protonated KPS-A and genistein were targeted for fragmentation, and the most stable and abundant ions in the product ion scan of KPS-A and genistein were *m/z* 585.5 and 158.8, respectively. Subsequently, the mass transitions were monitored at *m/z* 749.4→585.5 for KPS-A and *m/z* 268.9→158.8 for genistein. Other conditions such as ion spray voltage, curtain gas pressure, nebulizer gas pressure, heater gas pressure, source temperature and collision energy were further optimized to improve the sensitivity and response stability of KPS-A.

During the optimization of chromatographic conditions, it was investigated that KPS-A was extensively retained on several kinds of columns due to its strong lipophilicity. To achieve symmetric peak shapes and short chromatographic running times, the mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used on a Zorbax XDB C_{18} column. And finally a gradient elution program was utilized as follows: 0.00 min 10% B, 0.30 min 10% B, 1.00 min 80% B, 2.50 min 95% B, 2.51 min 10% B, 3.50 min 10% B. Under the present chromatographic conditions, symmetric peak shapes of KPS-A and genistein were obtained, and the running time is only 3.5 min.

Mass chromatograms of KPS-A and IS obtained by extraction of blank rat plasma, blank plasma spiked with KPS-A and IS, and actual unknown plasma samples obtained in rats after intravenous and oral injection of KPS-A are shown in Fig.2. KPS-A and genistein were eluted at 2.09 and 2.01 min, respectively. Each analytical run was achieved within 3.5 min. No endogenous or extraneous peaks interfering with the analytes were observed.

An internal standard is usually required in LC-MS/MS analysis in order to eliminate the effects from matrix and the extraction efficiency. In this study, genistein, a readily available compound, was selected as the IS, which displays similar chromatographic retention behavior (t_R = 2.01 min) with KPS-A and high extraction efficiency (>80%).

3.2 Assay validation

LC-MS/MS results may be adversely affected by lack of specificity and selectivity due to ion suppression caused by the sample matrix effect. In present assay, the mean absolute matrix effect value obtained was 94.7% for KPS-A (Table 2), which indicated that ion suppression or enhancement from plasma matrix was acceptable for this current analytical method, which is in agreement with international guidelines [27, 28].

The recoveries were determined in five replicates at low, medium and high concentrations of KPS-A. The results are summarized in Table 2. The mean recoveries of KPS-A were all above 90%. The data indicated that the recoveries of KPS-A from rat plasma were concentration-independent in the concentration range evaluated and the recoveries were acceptable for the pharmacokinetic analysis.

The calibration curves ranging from 0.50 to 500 ng mL^{-1} were linear for the analysis of KPS-A in rat plasma. The slopes, intercepts, and correlation coefficients of the regression equations were determined by least squares linear regression using a weight-factor of $1/x^2$. Typical equations for the standard curves were $y = 2.15\times10^{-4}\ x + 3.20\times10^{-5}$ (r=0.9974). Deviations were within ±15% for all regression equations. The lower limit of quantification (LLOQ) was 0.50 ng mL^{-1} for KPS-A in rat plasma.

Table 3 summarized the intra-and inter-day precisions and accuracies of KPS-A at different concentration levels. As shown in Table 3, the intra- and inter-day accuracies of KPS-A (% RE) were within the range of ±15%. The intra- and inter-day precisions (% RSD) were all less than 15%. The results demonstrated that the values were all within the acceptable range and the method was proved to be accurate and precise.

The stability results were presented in Table 4. The results indicated that KPS-A at the three concentrations tested had acceptable stabilities after three cycles of freeze-thaw, at room temperature for 24 hr and at -80°C for 1 month with the % RE values being within ± 15%.

In conclusion, the present method was demonstrated to have satisfactory performance in agreement with international guideline for the separation and determination of KPS-A in rat plasma [26, 27]. The LLOQ of the developed assay (0.50 ng mL^{-1}) was sufficient to characterize the pharmacokinetics of KPS-A at intravenous dose of 5 mg kg^{-1} and oral dose of 100 mg kg^{-1}.

3.3 Application of method

The developed assay was applied to an intravenous and oral study of the pharmacokinetics of KPS-A in rats. The plasma concentration-time profiles of KPS-A in rats are shown in Fig.3 and the main pharmacokinetic parameters of KPS-A after intravenous and oral administration are presented in Table 5. After intravenous administration of KPS-A at the dose of 5 mg kg^{-1}, the elimination half-life ($t_{1/2}$) value was estimated to be 3.70 ± 0.70 hr, and the mean area under the plasma concentration-time curve from time zero to the last measurable plasma concentration point (AUC_{0-t}) and the mean area under the plasma concentration-time curve from time zero to time infinity ($AUC_{0-\infty}$) values were 110379 ± 58609 and 110409 ± 58589 ng mL^{-1} × hr, respectively. Clearance (CL_z), mean residence time ($MRT_{(0-\infty)}$) and volume of distribution (V_z) values were estimated to be 0.06 ± 0.03 L hr^{-1} kg^{-1}, 4.79 ± 2.08 hr and 0.30 ± 0.20 L kg^{-1}, respectively. After oral administration of KPS-A at the dose of 100 mg kg^{-1}, KPS-A was rapidly absorbed, reaching mean C_{max} of 17.0 ± 5.90 ng mL^{-1} at T_{max} of 1.33 ± 0.58 hr. The mean AUC_{0-t} and $AUC_{0-\infty}$ values were 112 ± 37.2 and 137 ± 47.3 ng mL^{-1} × hr, respectively. The oral absolute bioavailability (F) of KPS-A was calculated to be 0.006 ± 0.002% with an elimination half-life ($t_{1/2}$) value of 18.4 ± 16.1 hr, suggesting its poor absorption and/or strong metabolism *in vivo*. Significant first pass effect and poor permeability through the intestinal epithelial membrane after oral administration might be responsible for the low bioavailability of this compound.

It is predicted that KPS-A might be metabolized into its aglucone hederagenin via the dissociation of two sugars (rhamnose and arabinose) from the parent molecule. As a result, hederagenin, the aglucone of KPS-A had also been simultaneously determined in the rat plasma. However, hederagenin is not detected in all the rat plasma, indicating that KPS-A might not metabolize into hederagenin *in vivo*.

Overall, the oral absolute bioavailability (F) of KPS-A is very low (0.006 ± 0.002%), suggesting that KPS-A shows poor absorption and/or strong metabolism *in vivo*, which is now being further confirmed

through rat excretion experiments after oral and intravenous administration in our laboratory. In addition, it is indicated that parenteral administration route is first choice for KPS-A in order to improve its efficacy.

4. Conclusion

An accurate and sensitive LC-MS/MS assay was developed for the determination of KPS-A in rat plasma. The sample preparation involved a simple protein precipitation (PPT) procedure with methanol:acetonitrile (50:50, v/v). The LLOQ was 0.50 ng mL^{-1} using 25 μL of rat plasma. The assay showed a wide linear dynamic range of 0.50–500 ng mL^{-1}, with acceptable intra- and inter-day accuracy and precision. The developed and validated method has been successfully applied in the quantification and pharmacokinetic study of KPS-A in rats after intravenous and oral administration. The oral absolute bioavailability (F) of KPS-A was calculated to be 0.006 ± 0.002%, suggesting its poor absorption and/or strong metabolism *in vivo*.

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Figure Captions:

Fig.1. Chemical structures, full-scan product ion spectra of $[M-H]^-$ ions and fragmentation schemes for (A) KPS-A and (B) genistein (internal standard)

Fig.2. Typical MRM chromatograms of (A) blank rat plasma; (B) blank rat plasma spiked with KPS-A (0.50 ng mL^{-1}, LLOQ) and IS; (C) an unknown rat plasma sample collected at 2 hr after oral administration of 100 mg kg^{-1} KPS-A; and (D) an unknown rat plasma sample collected at 30 min after intravenous administration of 5 mg kg^{-1} KPS-A (200 fold dilution using blank rat plasma)

Fig.3. Mean plasma concentration-time profiles of KPS-A determined by LC-MS/MS method after intravenous and oral administration of KPS-A to rats. Each point represents mean ± SD (n=3)

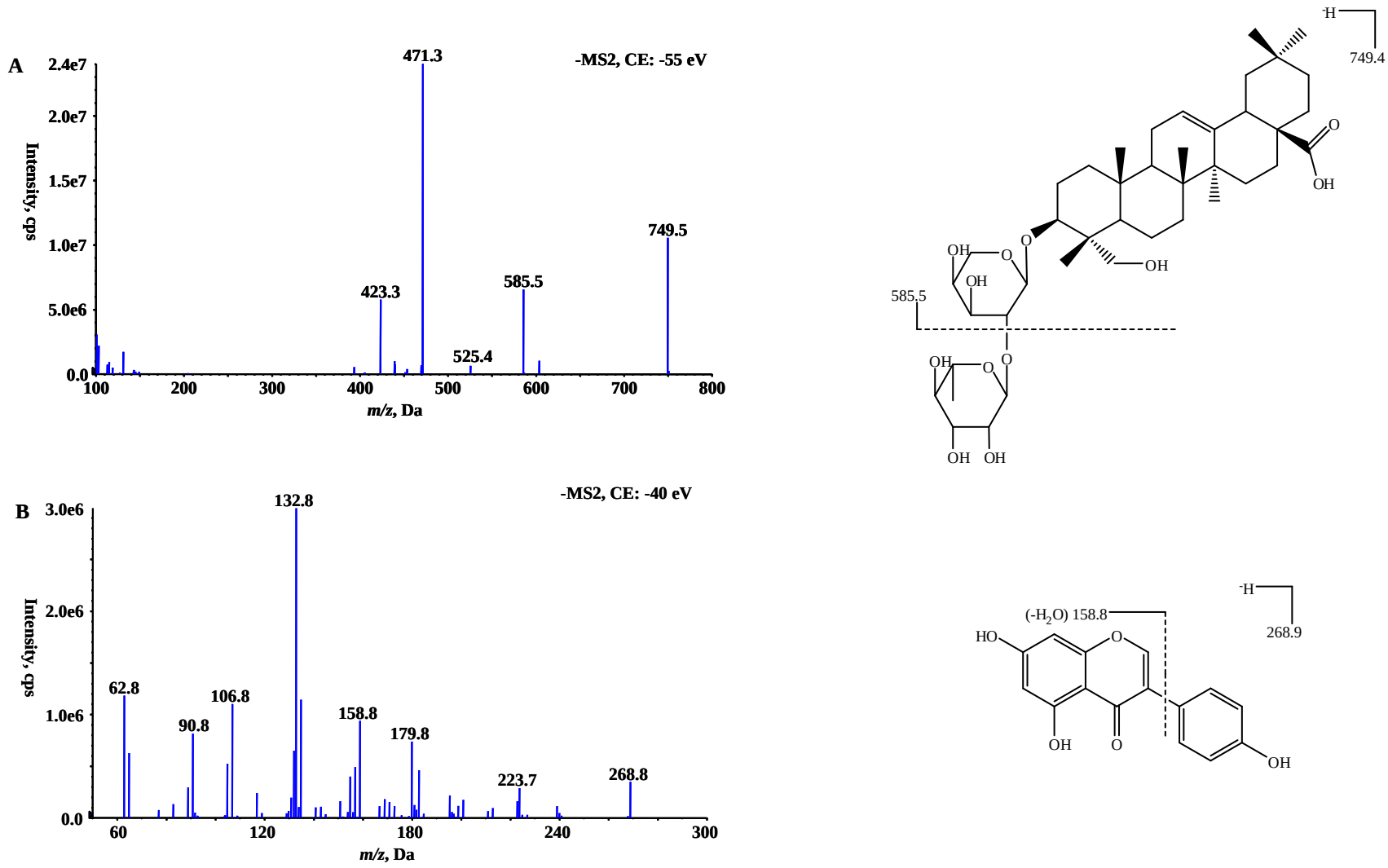
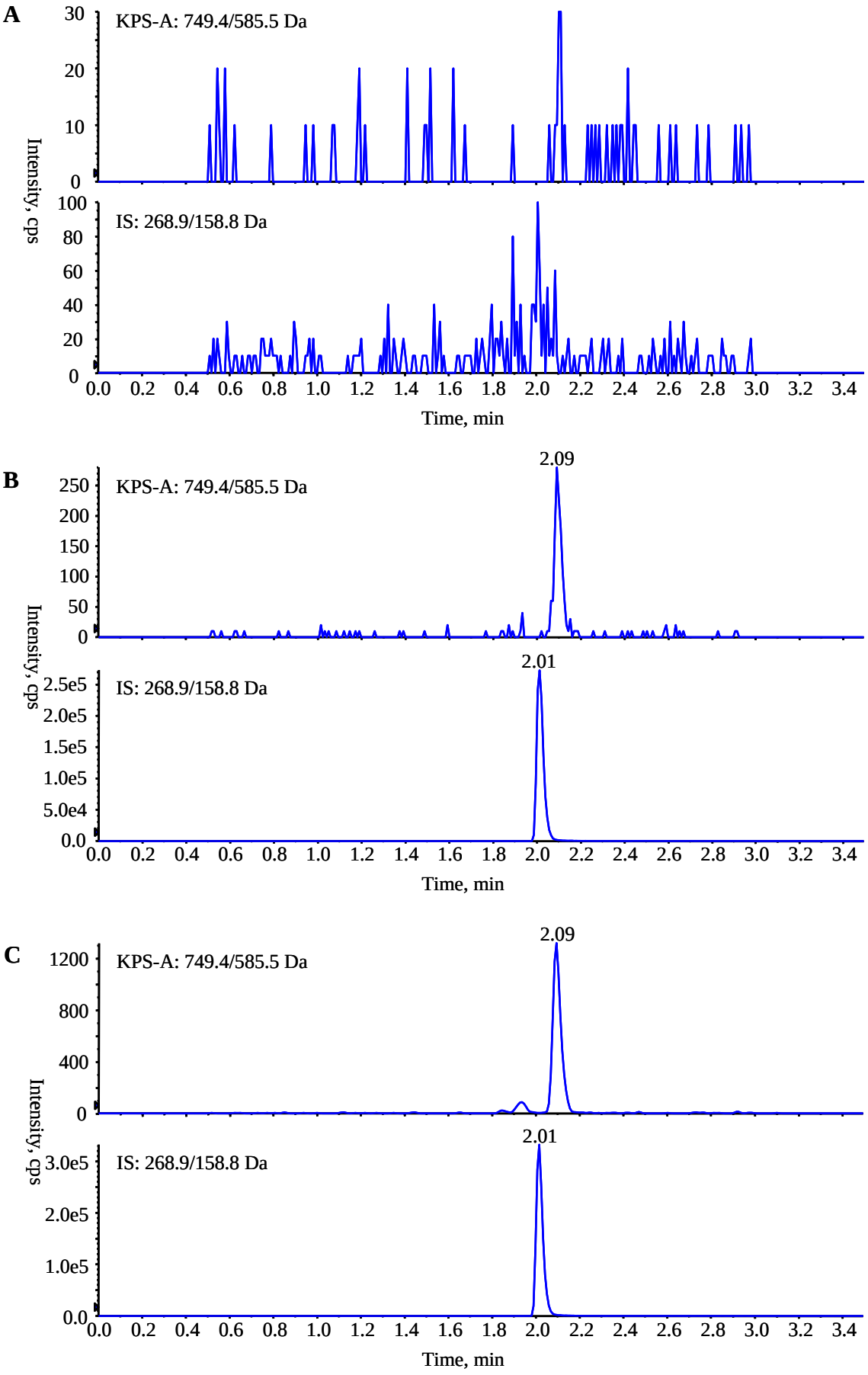
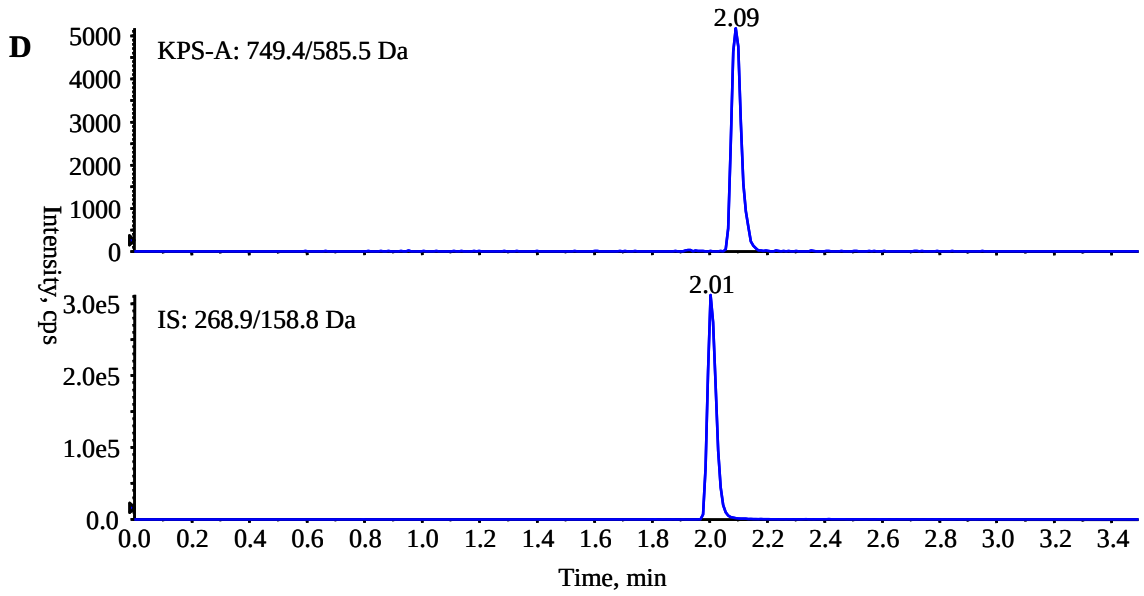


Fig.1. Chemical structures, full-scan product ion spectra of $[M-H]^-$ ions and fragmentation schemes for (A) KPS-A and (B) genistein (internal standard)





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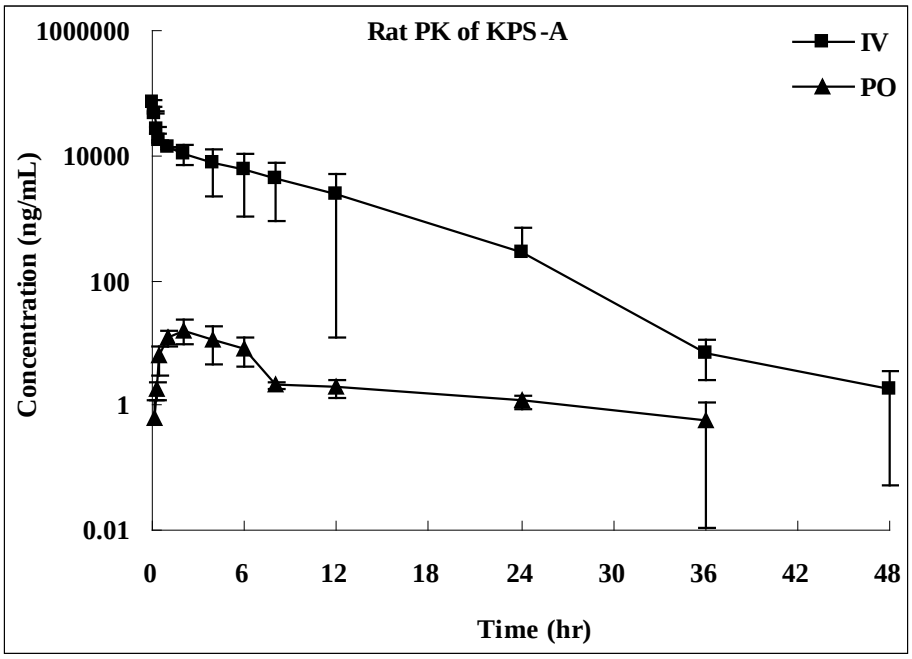

Fig.3. Mean plasma concentration-time profiles of KPS-A determined by LC-MS/MS method after intravenous and oral administration of KPS-A to rats. Each point represents mean ± SD (n=3)

Table Captions:

Table 1 MS parameters of KPS-A and genistein (IS) determined by LC-MS/MS

Table 2 Matrix effects and recoveries of KPS-A in rat plasma determined by LC-MS/MS (n=5)

Table 3 Intra- and inter-day accuracy and precision data (mean ± SD) for KPS-A in rat plasma determined by LC-MS/MS (n=5)

Table 4 Stability results of KPS-A in rat plasma under three different conditions determined by LC-MS/MS (n=5)

Table 5 Main pharmacokinetic parameters of KPS-A in rats determined after intravenous and oral administration of KPS-A (n=3, mean ± SD)

Table 1 MS parameters of KPS-A and genistein (IS) determined by LC-MS/MS

MS Parameters	KPS-A			Genistein
	For quantification	For confirmation		(IS)
Parent ion (m/z)	749.4	749.4	749.4	268.9
Product ion (m/z)	585.5	471.3	423.3	158.8
DP (V)	-167	-154	-158	-90
CE (eV)	-55	-62	-57	-40
CXP (V)	-10	-10	-10	-10
IonSpray voltage	-4200			
Curtain gas	15			
CAD	High			
Ion Source Gas 1	65			
Ion Source Gas 2	55			
Source temperature	550			

DP: Declustering potential; CE: collision energy; CXP: Cell exit potential; CAD: Collision-activated dissociation.

Table 2 Matrix effects and recoveries of KPS-A in rat plasma determined by LC-MS/MS (n=5)

Spiked concentration (ng mL^{-1})	Matrix effect (%)	RSD (%)	Recovery (%)	RSD (%)
1.00	94.5	6.8	93.2	2.6
20.0	95.7	4.6	94.4	0.6
400	93.8	4.2	92.9	1.4

Table 3 Intra- and inter-day accuracy and precision data (mean ± SD) for KPS-A in rat plasma determined by LC-MS/MS (n=5)

Batch	Item	Low 1.00 ng mL^{-1}	Medium 20.0 ng mL^{-1}	High 400 ng mL^{-1}
Day 1	Mean ± SD	1.00 ± 0.06	20.4 ± 1.87	401 ± 14.2
	RSD (%)	6.3	9.2	3.5
	Accuracy (%)	100.2	101.9	100.2
Day 2	Mean ± SD	1.03 ± 0.06	20.2 ± 0.91	404 ± 9.81
	RSD (%)	6.2	4.5	2.4
	Accuracy (%)	102.7	100.8	101.0
Day 3	Mean ± SD	0.97 ± 0.08	20.7 ± 1.12	403 ± 15.6
	RSD (%)	8.1	5.4	3.9
	Accuracy (%)	96.8	103.4	100.7
Inter-Day	Mean ± SD	1.00 ± 0.07	20.4 ± 1.30	403 ± 12.7
	RSD (%)	6.9	6.4	3.2
	Accuracy (%)	99.9	102.0	100.6

Table 4 Stability results of KPS-A in rat plasma under three different conditions determined by LC-MS/MS (n=5)

Stability conditions	Added Conc.	1.00 ng mL^{-1}	20.0 ng mL^{-1}	400 ng mL^{-1}
Three freeze–thaw cycles	Mean ± SD	1.01 ± 0.05	20.4 ± 0.92	407 ± 15.5
	RSD (%)	4.8	4.5	3.8
	Recovery (%)	101.1	101.8	101.8
Room temperature for 24 hr	Mean ± SD	0.93 ± 0.07	21.7 ± 0.52	427 ± 12.0
	RSD (%)	7.9	2.4	2.8
	Recovery (%)	93.1	108.5	106.7
Storage at −80°C for 1 month	Mean ± SD	0.93 ± 0.07	19.9 ± 0.17	383 ± 15.8
	RSD (%)	7.9	0.9	4.1
	Recovery (%)	93.1	99.5	95.7

Table 5 Main pharmacokinetic parameters of KPS-A in rats determined after intravenous and oral administration of KPS-A (n=3, mean ± SD)

Parameters	Unit	IV	PO
$AUC_{(0-t)}$	ng mL^{-1} × hr	110379 ± 58609	112 ± 37.2
$AUC_{(0-\infty)}$	ng mL^{-1} × hr	110409 ± 58589	137 ± 47.3
$MRT_{(0-t)}$	hr	4.77 ± 2.10	7.86 ± 1.48
$MRT_{(0-\infty)}$	hr	4.79 ± 2.08	18.8 ± 14.7
$t_{1/2z}$	hr	3.70 ± 0.70	18.4 ± 16.1
CL_z	L hr^{-1} kg^{-1}	0.06 ± 0.03	801 ± 324
V_z	L kg^{-1}	0.30 ± 0.20	18747 ± 11746
T_{max}	hr	-	1.33 ± 0.58
C_{max}	ng mL^{-1}	-	17.0 ± 5.90
F	%	-	0.006 ± 0.002