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Short communication

A sensitive liquid chromatography–electrospray tandem mass spectrometric method for lancemaside A and its metabolites in plasma and a pharmacokinetic study in mice

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ABSTRACT

A high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) method employing electrospray ionization (ESI) has been developed for simultaneous determination of lancemaside A (3-*O*-β-D-glucuronopyranosyl-3β, 16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester) and its metabolites in mouse plasma. When lancemaside A (60 mg/kg) was orally administered to mice, echinocystic acid was detected in the blood. T_{max} and C_{max} of the echinocystic acid were 6.5 ± 1.9 h and 56.7 ± 29.1 ppb. Orally administered lancemaside A was metabolized to lancemaside X (3β, 16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester) by intestinal microflora in mice, which was metabolized to echinocystic acid by intestinal microflora and/or intestinal tissues. Human intestinal microflora also metabolized lancemaside A to echinocystic acid via lancemaside X. These results suggest that the metabolism by intestinal microflora may play an important role in pharmacological effects of orally administered lancemaside A.

1. Introduction

Lancemaside A, 3-*O*-β-D-glucuronopyranosyl-3β, 16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl (1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester, is a triterpenoid saponin isolated from BuOH extract of the rhizome of *Codonopsis lanceolata* (family Campanulaceae) [1]. Triterpenoid saponins of the rhizome of *C. lanceolata*, which contain lancemaside A as a major compound, are identified by centrifugal partition chromatography and liquid chromatography–mass spectrometry [2–4]. The rhizome of *C. lanceolata* is been used in herbal medicines for inflammatory diseases such as bronchitis and cough in Asian countries [5–7]. Their saponins exhibit anti-inflammatory and anti-tumor effects [7,8]. We also reported that lancemaside A isolated from its BuOH fraction potently inhibited colitis via TLR-linked NF-κB activation in mice [9]. Similar to other saponins, such as ginsenoside Rb1, the absorption of orally administered lancemaside A from intestine into the blood may be difficult due to its hydrophilicity [10–13]. Thus, orally administered lancemaside A comes into contact with intestinal microflora in intestine and is metabolized to hydrophobic compounds and its metabolites may be absorbed into the blood.

Therefore, to understand its bioactive form, we performed a pharmacokinetic study of lancemaside A in mice.

2. Experimental

2.1. Chemicals, materials and reagents

Lancemaside A was isolated from *C. lanceolata* (CL) as previously reported by Joh et al. [9]. Compound K was isolated using the previously published method of Bae et al. [14]. β-D-Glucuronidase was purchased from Sigma (St Louis, MO, USA). Acetonitrile, methanol and formic acid (HPLC grade) were purchased from Samchun Chemicals (Pyeongtaek, Gyeonggi, Korea). All other reagents were of analytical grade.

2.2. Animals

Male ICR mice (24–28 g) were supplied from Orient animal breeding center (Seoul, Korea). All animals were housed in wire

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cages at 20–22 °C and 50 ± 10% humidity, fed standard laboratory chow (Samyang, Seoul, South Korea), and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

2.3. Preparation of lancemaside X, echinocystic acid and echinocystic acid 3-O-β-D-glucuronopyranoside (EAG)

For the preparation of lancemaside X (3β, 16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl (1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester), the reaction mixture containing 2 mL of lancemaside A (10 mg/mL) dissolved in distilled water and 5 mL of β-D-glucuronidase (0.5 mg/mL) dissolved in distilled water was incubated at 37 °C for 5 h. The reaction mixture (7 mL) was extracted with BuOH. The BuOH layer was evaporated *in vacuo*, dissolved in acetonitrile, and applied to MPLC to afford lancemaside X (4 mg).

For the preparation of echinocystic acid, lancemaside A (50 mg) in 5 mL H_2O was added to 6N HCl (5 mL) and refluxed 2 h. The mixture was extracted with $CHCl_3$. The chloroform layer was evaporated *in vacuo*, dissolved in acetonitrile, and applied to MPLC to afford echinocystic acid (16 mg).

For the preparation of EAG, lancemaside A (50 mg) in 5 mL H_2O was added to 5N NaOH (5 mL) and heated in a water bath at 70 °C. The mixture was extracted with BuOH. The BuOH layer was evaporated *in vacuo*, dissolved in acetonitrile, and applied to MPLC to afford EAG (18 mg). Chromatographic separation was carried out on a Ultra-Pak C18 column (300 mm × 37 mm, 50 μm, Yamazen Co. Ltd., Japan). The linear-gradient of elution used is as follows: 0–4 h, 40–95% mobile phase A. The mobile phase A is acetonitrile whereas the mobile phase B is water, and pumped at flow rate of 4 mL/min.

2.4. Preparation of standard solutions

Stock solutions were prepared by dissolving lancemaside A, lancemaside X, EAG and echinocystic acid in acetonitrile and MeOH solvent (2:1, v/v) at a concentration of 1 ppm, and were further diluted with the same solvent to concentrations of 100 and 15 ppb. All working solutions were stored in a refrigerator (−20 °C).

2.5. HPLC–MS/MS instrumentation

LC–MS/MS analyses were performed on the Agilent G6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source, coupled with an HPLC Agilent 1200 series (Agilent, CA, USA). Chromatographic separations of the sample were performed on a ZORBAX Eclipse XDB-C18 column (50 mm × 2.1 mm, i.d., 1.8 μm, Agilent). For elution, a linear gradient was applied from CH_3CN–H_2O (40:60, v/v) to CH_3CN–H_2O (95:5, v/v) for 10 min. The solvent flow rate was 0.3 mL/min. Mass spectra were acquired in ESI mode using nitrogen gas at a temperature of 350 °C, a flow rate 10 L/min, a nebulizer pressure of 45 psi, quadrupole temperature of 30 °C, and a capillary voltage of 4000 V. Samples (3 μL) were injected into the column using an autosampler. Quantitation was performed using the MRM of the transitions of m/z 1189.6 → 647.1 for lancemaside A, m/z 1014.6 → 471.2 for lancemaside X, m/z 647.3 → 407.3 for EAG and m/z 471.4 → 407.3 for echinocystic acid in negative mode.

2.6. Preparation of samples

Seven hundred microliters of mouse blood were centrifuged for 10 min at 4000 × *g*. To deprotonize the plasma, 300 μL acetonitrile and MeOH solution (2:1, v/v) were added to 300 μL supernatant, vortexed for 2 min and centrifuged for 10 min at 13,000 × *g*. For analysis, the LC–MS/MS system (Agilent, CA, USA) was used.

2.7. Method validation

Intra-day assays were performed using three replicates during 1 day, and inter-day assays were performed on 3 separate days. The precision was calculated as the RSD and the accuracy was expressed as the relative error [RE (calculated concentration − nominal concentration)/(nominal concentration) × 100%]. Recoveries and matrix effects at four QC levels were determined by comparing the peak areas of the pure solution with the peak areas of pre-extraction and post-extraction plasma blanks spiked with an equivalent concentration using three replicates. The short-term, storage term and freeze/thaw stability in mouse plasma was studied at 100 ppb using three replicates. All stability studies were evaluated by comparison of plasma samples stored under different conditions with freshly prepared samples.

2.8. Pharmacokinetic study

The mice were divided into 11 groups of four mice per group. Each mouse was orally administered lancemaside A at a dose of 60 mg/kg. The mice were exsanguinated from the heart at 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after lancemaside A administration, group by group.

2.9. Analysis of digestive tract contents

The mice were divided into 5 groups of four mice per group. Each mouse was orally administered lancemaside A at a dose of 60 mg/kg. The mice were anesthetized with ether and killed at 0, 1, 3, 5, and 8 h after lancemaside A administration. The stomach, small intestine, cecum and colon were quickly removed, opened and the contents collected, group by group. The contents of each mouse were suspended in 10-fold volume of saline in a cooled tube, and centrifuged at 250 × *g* for 5 min. The supernatants were extracted with BuOH, evaporated to dryness and resuspended in 1 mL of MeOH. ESI-MS analyses were performed on a LCQ DECA XP MS (Thermo Finnigan, CA, USA) equipped with an electrospray ion source. All ion trap analyzer parameters were optimized according to the manufacturer's instructions. In ESI-MS experiments, the spray voltage was 4.5 kV in positive mode and −4 kV in negative mode under N_2 sheath gas flow at 50 arbitrary units. The capillary temperature was maintained at 275 °C. Two microliters of samples were injected into the column. Total ion chromatograms from m/z 150 to 2000 in ESI positive and negative modes were obtained. For tandem mass spectrometry, the maximum ion injection time, activation time, and isolated ion width were set to 500, 30 and 2.0 ms, respectively. The collision energy with helium was set to 30% of the radio frequency (5 V) applied to the ion trap analyzer.

2.10. Metabolism of lancemaside A by human fecal suspension

A reaction mixture containing 500 μL of lancemaside A (1 mg/mL) dissolved in distilled water and 4.5 mL of human fecal bacterial suspension was anaerobically incubated at 37 °C for 24 h. The reaction mixture (0.5 mL) was extracted with BuOH at 0, 1, 3, 5, and 8 h after incubation, evaporated to dryness and resuspended in 0.5 mL of MeOH for analysis with LC–MS/MS. LC/MS/MS analyses were performed on Agilent G6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source, coupled with an HPLC Agilent 1200 series. Chromatographic separation of the sample was performed on a ZORBAX Eclipse XDB-C18 column

Table 1
Accuracy and precision of lancemaside A, lancemaside X, echinocystic acid 3-*O*-β-D-glucuronopyranoside (EAG), and echinocystic acid.

	Added concentration (ppb)	Detected concentration (ppb)	Intra-day RSD (%)	Inter-day RSD (%)	RE (%)
Lancemaside A	15	15.0	6	3	0
	100	104.7	2	6	5
	1000	964.0	5	3	−4
Lancemaside X	15	14.7	5	5	−2
	100	104.2	8	3	4
	1000	985.4	3	6	−1
EAG	15	14.6	2	3	−3
	100	107.6	5	10	8
	1000	1056.5	9	2	6
Echinocystic acid	15	15.1	1	1	1
	100	98.3	3	1	−2
	1000	1036.9	2	0	4

(50 mm × 2.1 mm, i.d., 1.8 μm, Agilent). For elution a linear gradient was applied from CH_3CN–H_2O (20:80, v/v) to CH_3CN–H_2O (95:5, v/v) for 13 min. The solvent flow rate was 0.3 mL/min. Mass spectra were acquired in ESI mode using nitrogen gas at a temperature of 350 °C, a flow rate of 10 L/min, nebulizer pressure of 45 psi, a quadrupole temperature of 30 °C, and a capillary voltage of 4000 V. Sample (2 μL) was injected into the column using an autosampler. Total ion chromatograms from *m*/*z* 400 to *m*/*z* 1500 in MS/MS positive and negative modes were obtained.

For the preparation of human fecal suspensions, human stool (5 g) was suspended in 50 mL of saline and centrifuged at 500 × *g* for 10 min. The supernatant was precipitated at 10,000 × *g* for 30 min and washed twice with saline. The precipitate (260 mg) was suspended in 52 mL of 10 mM phosphate buffer (pH 6.5) and used as a fecal sample.

3. Results and discussion

3.1. Method validation

Lancemaside A, lancemaside X and echinocystic acid 3-*O*-β-D-glucuronopyranoside gave a fairly strong mass response in positive ESI mode, while echinocystic acid gave a fairly strong mass response in negative ESI mode. The ion peaks of lancemaside A and its metabolites were difficult to find. Overall, lancemaside A, lancemaside X, EAG and echinocystic acid gave a strong mass response in negative electrospray ionization (ESI) mode. By negative ESI, lancemaside A, lancemaside X, EAG, echinocystic acid and IS formed predominantly deprotonated molecular ions $[M-H]^-$ at *m*/*z* 1189, *m*/*z* 1014, *m*/*z* 647, *m*/*z* 471, and *m*/*z* 622 in full scan mass spectra, respectively. We used aqueous acetonitrile as the mobile phase due to their insolubility in methanol.

At an earlier stage of method development, several liquid–liquid extraction (LLE) methods were investigated. The recoveries of ESI, lancemaside A, lancemaside X, EAG, and echinocystic acid were below 30% when ethyl acetate, diethyl ether, or *n*-butanol was used as the extraction solvent. The high hydrophilicity of lancemaside A, lancemaside X and EAG might have made them difficult to extract from plasma by conventional LLE. Protein precipitation (PPT) often provides higher recovery compared with LLE for compounds with high hydrophilicity. A simple and rapid PPT protocol was developed that used acetonitrile–methanol (2:1) mixture as a deproteinizing solvent. Adding 300 μL water to 300 μL supernatant yielded a symmetrical peak shape and the least dilution for ESI of lancemaside A, lancemaside X, EAG, and echinocystic acid.

The selectivity of the method was examined by comparing blank plasma and spiked plasma ($n = 4$). Under the above conditions, the retention time was 0.46 min for lancemaside A, 1.15 min for lancemaside X, 0.61 min for EAG, 4.74 min for echinocystic acid and 2.16 min for the IS.

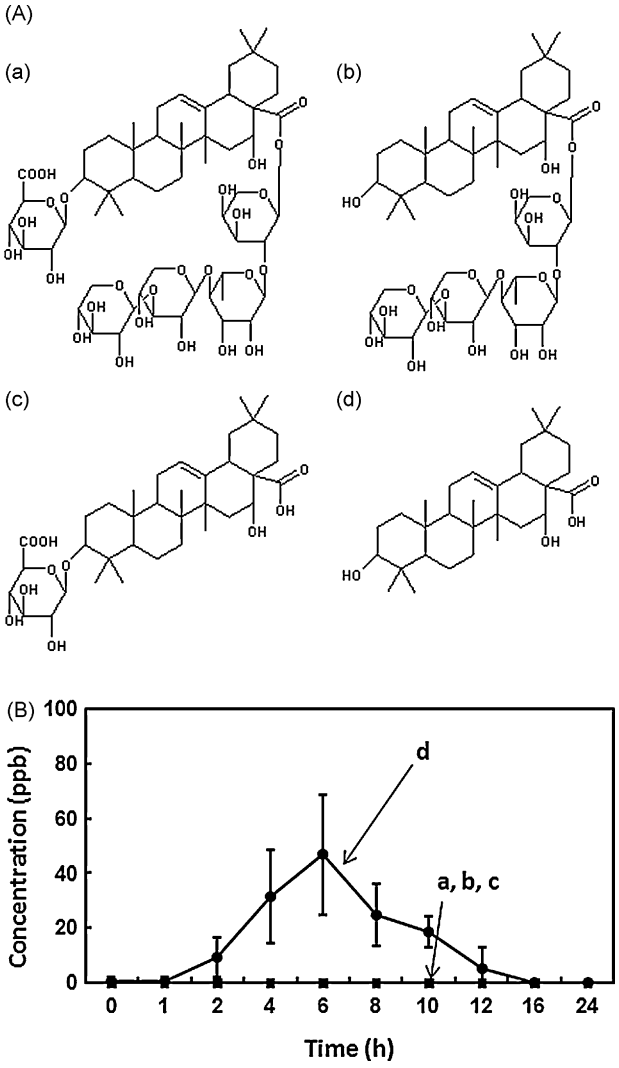

Fig. 1. Structures of lancemaside A and its metabolites (A) and mean plasma concentration–time profile of lancemaside A and its metabolites in mice after oral administration of lancemaside A (60 mg/kg) (B). Lancemaside A (a, closed squre), lancemaside X (b, closed diamond) and echinocystic acid 3-*O*-β-D-glucuronopyranoside (EAG, c, closed triangle) were not detected, but echinocystic acid (d, closed circle) was detected (each point represents mean ± SD, $n = 4$).

The linearity of the five-point calibration curves was good for LC–MS/MS of the four compounds in the concentration range of 15–10,000 ppb. The equations of the calibration curves were $y = 0.0292x - 1.8770$ ($r^2 = 0.9997$), $y = 0.0125x - 0.9062$ ($r^2 = 0.9996$), $y = 0.0069x + 1.6748$ ($r^2 = 0.9993$) and $y = 0.0789x - 4.1193$ ($r^2 = 0.9998$) for the four analytes,

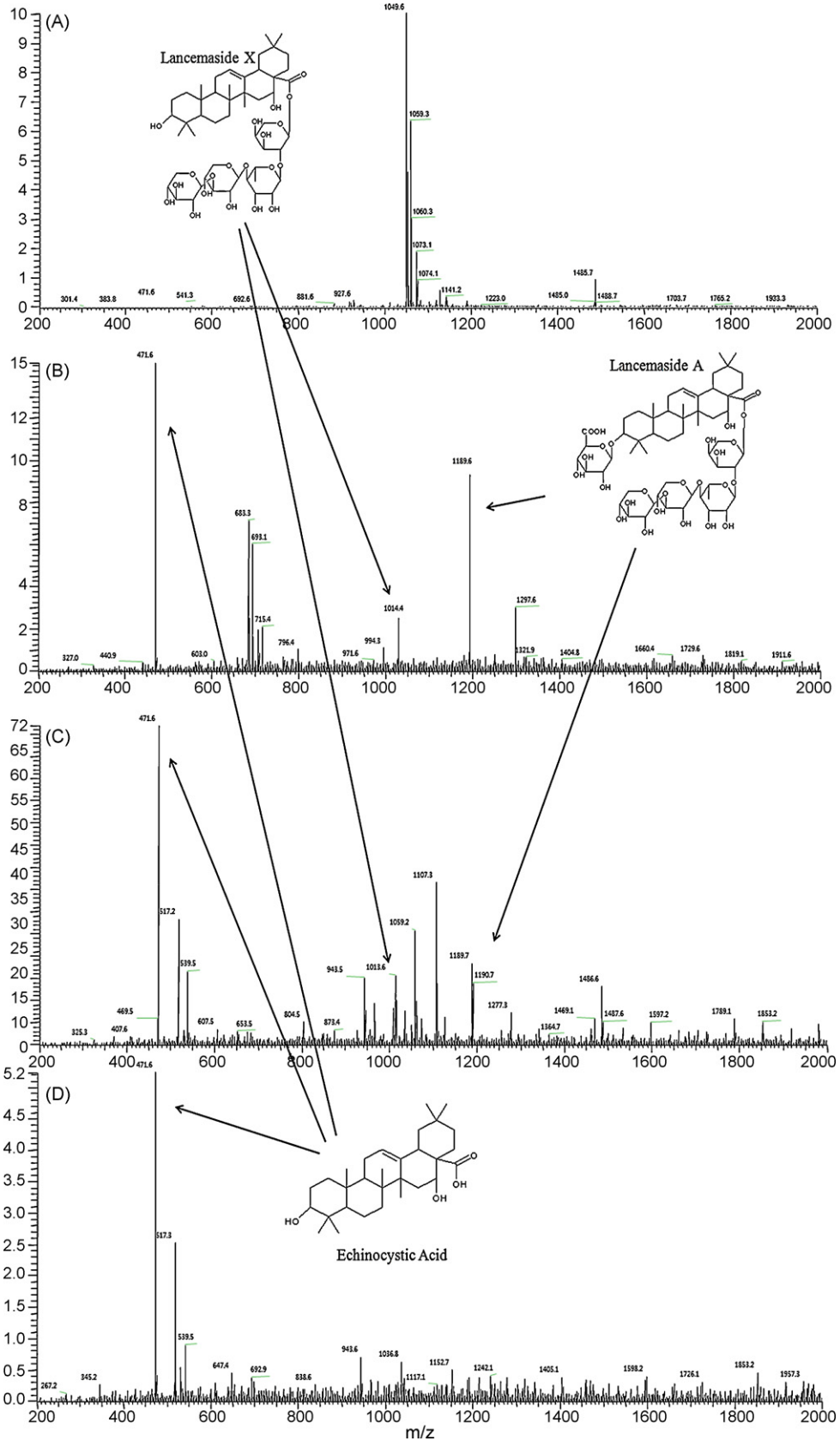

Fig. 2. ESI-MS analysis of lancemaside A and its metabolites in the digestive tract contents of mice orally administered lancemaside A. Three main peaks were detected: the ion peak at *m*/*z* 1189 is lancemaside A, another ion peak at *m*/*z* 1014 is lancemaside X, the other ion peak at *m*/*z* 471 is echinocystic acid (5 h after oral administration of lancemaside A; A, stomach contents; B, small intestine contents; C, cecum contents; D, colon contents).

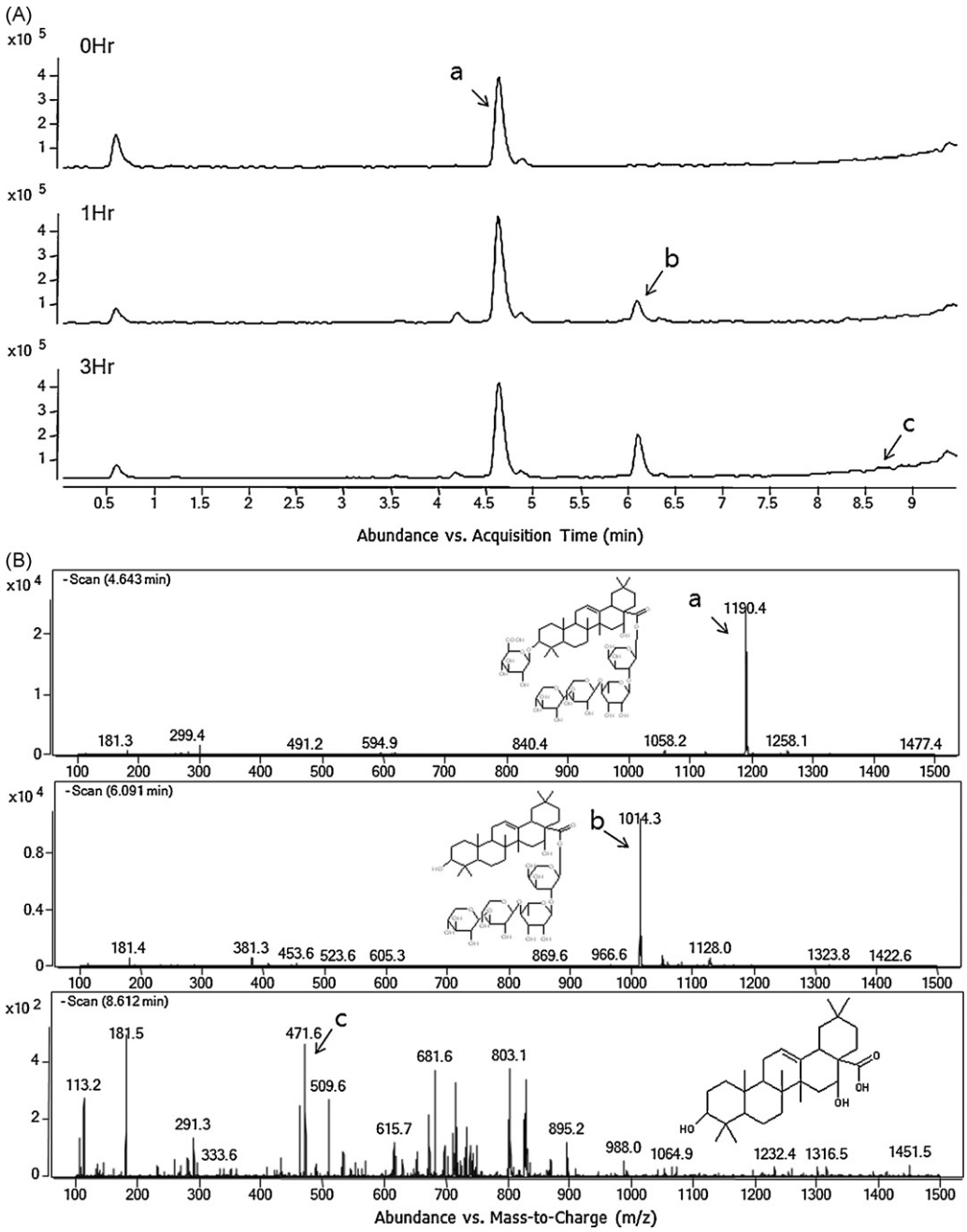

Fig. 3. Metabolism of lancemaside A by human fecal suspension. (A) LC–MS/MS chromatogram of the reaction mixture incubated with human fecal microflora (before incubation, 1 h after incubation and 3 h after incubation). The reaction mixture was extracted with BuOH and analyzed by LC–MS/MS. (B) The mass spectra of lancemaside A and its main metabolites.

respectively, where y represents the ratio of the lancemaside A, lancemaside X, EAG or echinocystic acid peak area to that of IS and x represents their plasma concentrations. The LLOQs were sufficient for pharmacokinetic studies of these compounds in mice.

Good performance with low deviation and consistent accuracy for four compounds was observed at three QC levels of 15, 100 and 1000 ppb during the validation. As shown in Table 1, the intra- and inter-day precision were less than 9 and 10%, respectively, and the accuracy was within the range of 98.3–105.7% during the validation, indicating a precise and accurate method for the determination of lancemaside A, lancemaside X, EAG and echinocystic acid in mouse plasma.

The extraction recoveries for lancemaside A, lancemaside X, EAG and echinocystic acid in mouse plasma were >81% at 100 ppb (n = 4), and that for the IS (100 ppb) was 76% (n = 4). The mean absolute matrix effects of lancemaside A, lancemaside X, EAG and echinocystic acid were −2.0 to 1.6%; their relative matrix effects were 4.5–7.2% (data not shown). The stability experiments demonstrated that lancemaside A, lancemaside X, EAG and echinocystic acid were stable in mouse plasma after 24 h at room temperature, after 24 h at 10 °C in an autosampler, after 18 days storage at −20 °C and after three freeze/thaw cycles.

3.2. Pharmacokinetic study

The method described above was successfully applied to a pharmacokinetic study in which plasma concentrations of lancemaside A were determined for 24 h after oral administration (60 mg/kg,

Table 2
Pharmacokinetic parameters of echinocystic acid after oral administration of lancemaside A to mice.

Parameter	Oral administration ($n = 4$)
AUC (0–t) (ppb h)	294.0 ± 124.8
AUC (0–∞) (ppb h)	294.2 ± 124.7
$T_{1/2}$ (h)	2.8 ± 0.8
T_{max} (h)	6.5 ± 1.9
C_{max} (ppb)	56.7 ± 29.1
CL (L/h kg)	0.2 ± 0.1
Vd (L/kg)	1.0 ± 0.5

$n = 4$). The pharmacokinetic profiles of lancemaside A, lancemaside X, EAG and echinocystic acid are shown in Fig. 1 and the main pharmacokinetic parameters are given in Table 2. Lancemaside A, lancemaside X and EAG were not detected in plasma, although echinocystic acid was well detected. T_{max} of echinocystic acid was 6 h after oral administration of lancemaside A. The area under the serum concentration time curve (AUC) and the peak concentration (C_{max}) of echinocystic acid were 273.38 ppb h and 46.82 ppb, respectively. These findings suggest that orally administered lancemaside A might be metabolized to echinocystic acid by intestinal microflora and then absorbed into the blood.

3.3. Metabolism of lancemaside A by intestinal microflora

To confirm the metabolism of lancemaside A in a digestive tract of mice, we measured the mass spectra of the contents of stomach, small intestine, cecum and colon. Lancemaside A was detected in stomach 1 h after its oral administration, although lancemaside X, EAG and echinocystic acid were not detected (Fig. 2). However, 5 h after its oral administration, lancemaside A, lancemaside X and echinocystic acid level were detected in small intestine and cecum. In colon, only echinocystic acid was detected by ESI-MS. However, small intestinal and colonic tissue homogenates did not hydrolyze lancemaside A to lancemaside X, but did produce EAG. These findings suggest that orally administered lancemaside A may be quickly metabolized to lancemaside X by intestinal microflora, and then lancemaside X metabolized to echinocystic acid in the intestine by intestinal microflora and/or intestinal tissues.

To understand whether lancemaside A can be metabolized by human intestine, we anaerobically incubated lancemaside A with human fecal microflora and investigated its metabolite(s) by LC–MS/MS (Fig. 3). Lancemaside A was metabolized to two metabolites of MS peak at $m/z = 1014$ $[M-H]^-$ and 471 $[M-H]^-$. When the metabolism of lancemaside A in the human fecal specimens was assayed, lancemaside A was metabolized to echinocystic acid *via* lancemaside X acid, as in the mouse digestive tract. This finding suggests that orally administered lancemaside A is metabolized to echinocystic acid *via* lancemaside X in intestine and that the metabolite, echinocystic acid, may be absorbed into the blood.

4. Conclusion

In this study, we developed a rapid, sensitive and selective LC–MS/MS method and validated its use for the determination of lancemaside A, lancemaside X, EAG, and echinocystic acid in mouse plasma. When lancemaside A (60 mg/kg) was orally administered to mice, echinocystic acid was detected in the blood, but lancemaside A, lancemaside X and EAG were not detected. T_{max} and C_{max} of echinocystic acid were 6.5 ± 1.9 h and 56.7 ± 29.1 ppb, respectively. Orally administered lancemaside A was metabolized to lancemaside X by intestinal microflora in mice, lancemaside X was then metabolized to echinocystic acid by intestinal microflora and/or intestinal tissues, and echinocystic acid was absorbed into the blood. Human fecal microflora lancemaside A also metabolized lancemaside A to echinocystic acid. These results suggest that metabolism by intestinal microflora may play an important role in the pharmacological effects of orally administered lancemaside A.

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