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Pharmacokinetics, Tissue Distribution, and Tentative Metabolite Identification of Sauchinone in Mice by Microsampling and HPLC-MS/MS Methods

You-Jin Kim,[#] Seung Yon Han,[#] Jun Su Seo, Young-Won Chin, and Young Hee Choi*

College of Pharmacy and BK21 PLUS R-FIND Team, Dongguk University; Seoul, 32 Dongguk-lo, Ilsandong-gu, Goyang, Gyeonggi-do 410–820, Republic of Korea.
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Sauchinone, a biologically active lignan found in *Saururus chinensis* (Saururaceae), exerts various biological activities against jaundice, inflammatory disease, hepatic steatosis, and oxidative injury. Despite its diverse applications, there exists some information about sauchinone's pharmacokinetics but its tissue distribution, metabolism, and tentative metabolites have not been reported yet. Thus we investigated the pharmacokinetics of sauchinone in mice using microsampling and HPLC-MS/MS methods. Sauchinone presented linear pharmacokinetics at intravenous doses 7.5–20 mg/kg and oral doses 20–500 mg/kg. However, the metabolism of sauchinone was saturated and this agent presented nonlinear pharmacokinetics at 50 mg/kg in the intravenous study. At sauchinone 20 mg/kg the *F* of sauchinone was 7.76% of the oral dose despite that 77.9% of sauchinone was absorbed. This might be due to extensive metabolism of sauchinone in S9 fractions of liver and small intestine. Tentative metabolites of sauchinone by oxidation, dioxidation, methylation, demethylation, dehydrogenation, or bis-glucuronide conjugation were detected in plasma and S9 fractions of liver, intestine, and kidney. The distribution of sauchinone was considerably high (tissue-to-plasma (T/P) ratios, >1) in liver, small intestine, kidney, lung, muscle, fat, or mesentery after intravenous and oral administration and in stomach and large intestine only after oral administration. The protein binding value of sauchinone was 53.0%. These pharmacokinetic data of sauchinone provide an important basis for preclinical applications and experimental methods can be adjusted to evaluate the pharmacokinetics of natural products in mice.

Key words sauchinone; pharmacokinetics; metabolite identification; *Saururus chinensis*; mouse

Pharmacokinetic studies to investigate the absorption, distribution, metabolism, and excretion (ADME) of pharmacologically active compounds of interest are essential in the preclinical and clinical process[1,2] because ADME of compounds are fundamental to therapeutic outcome in relation with the efficacy and safety.[3] Along with the usages of a large number of herbal products as adjuvant or alternative medicines, the investigation of pharmacokinetic properties in herbal products has been demanding.[4–7] In addition, the tissue distribution and metabolism studies are meaningful to elucidate the pharmacological effects relying on the specific delivery and affinity of compounds to various tissues, extensive metabolizing organs, tentative metabolites identification.[7–10] At these points, the inevitable and integral reasons for pharmacokinetic investigations using mice, not like rats, are as followings: it is arduous to secure a sufficient amount of herbal product using rats and the use of various knockout or xenografted mouse models to demonstrate the pharmacological activities of herbal compounds is increasing in preclinical investigations[11,12] in terms of the different physiological and pathological conditions between mice and rats.[13,14]

Above all, the validated analytical method and an accurate and minimum blood sampling technique using mice are necessary to achieve the pharmacokinetic profiles in herbal products.[6–8] In light of a small volume of blood in mouse, only $<10\,\mu$L of blood collecting at each time looks appropriate and a microsampling system make it possible the sequential multi-blood sampling with accurate time point and blood volume from each mouse. Also an advanced analytical methods using high-performance liquid chromatography tandem mass-spectrometry (HPLC-MS/MS) allow to analyze a parent compound and probable metabolites in these biological samples with sufficient limit of quantitation.[6–8,15]

Sauchinone is a biologically active lignan found in *Saururus chinensis* Hort. ex Loudon (Saururaceae), a plant with a long history of medical use for treating fever, jaundice, edema, and inflammatory disease. It is also used as a protectant against hepatic steatosis and oxidative injury and as an AMP-activated protein kinase (AMPK) activator.[16–19] Previously, the analytical method and pharmacokinetics of sauchinone after intravenous administration in rats were reported based on the plasma concentrations of sauchinone.[20] However, the bioavailability, tissue distribution, metabolism, prediction of probable metabolites and protein binding of sauchinone have not been reported yet. For a popular trend of oral administration in herbal products by convenience and safety, the pharmacokinetic characteristics including the bioavailability of sauchinone after oral administration are required. Moreover, in the process of our preliminary oral study, the improvement of quantitation limit and simultaneous analysis of sauchinone and probable metabolites seemed to be necessary and it looks urgent in pharmacokinetics using mouse. Therefore, for the first time, we carried out the pharmacokinetic characteristics at various doses of sauchinone tissue distribution, metabolism (including tentative metabolite identification) and protein binding of sauchinone in mice as an example to confirm the

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: choiyh@dongguk.edu

evaluating tools for the ADME of herbal products in preclinical levels. Also the analytical validation of sauchinone and probable metabolite identification in mouse were conducted.

MATERIALS AND METHODS

Chemicals and Reagents Sauchinone (>99.0% purity) and gartanin (>98.5% purity; internal standard (IS) for HPLC-MS/MS) were extracted and purified according to a method reported previously.[19,21] Dextran, the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH; as a tetrasodium salt), uridine diphosphate glucuronic acid (UDPGA; as a trisodium salt), 3′-phosphoadenosine 5′-phosphosulfate (PAPS; as a lithium salt) and Tris buffer were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Polyethylene glycol 400 (PEG 400) was obtained from Showa Chemical Co. (Tokyo, Japan). Methanol, acetonitrile, formic acid, and distilled water were purchased from Fisher Scientific Co. (Seoul, South Korea). All other chemicals and reagents used were of analytical grade.

Animals The protocols for the animal studies (No. 2012–0673, July 2012) were approved by the Institute of Laboratory Animal Resources of Dongguk University-Seoul (Gyeonggi-do, South Korea). Male ICR mice (7–8 weeks old; weight, 20–30 g) were purchased from Charles River Co., Korea (Orient, Seoul, South Korea). Mice were acclimated for 1 week before starting the study. Upon arrival, animals were randomized and housed at three per cage under strictly controlled environmental conditions (20–25°C and 48–52% relative humidity). A 12-h light/dark cycle was used at an intensity of 150–300 lux.

Determination and Validation of Sauchinone Using the HPLC-MS/MS-Electrospray Ionization (ESI) Method To characterize the product ions of sauchinone and the IS, 0.5 μg/mL solutions of each compound were separately injected into the mass spectrometer at 10 μL/min. All analytics were performed using an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) in the multiple reaction monitoring (MRM) mode with an ESI interface for positive ions ($[M+H]^+$). The compounds were separated on a reverse-phase C_{18} column (Cadenza CD-C18, 2 mm×75 mm i.d., 3-μm particle size; Imtakt, San Diego, CA, U.S.A.) with an isocratic mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (35 : 65, v/v). The mobile phase was eluted at a flow rate of 0.30 mL/min. The column oven was maintained at 30°C.

The turbo ion-spray interface was operated in positive ion mode at an ion source voltage of 5500 V and a temperature of 500°C. The operating conditions, which were optimized by flow injection of a mixture of all analytes, were nebulizing gas flow, 50 psig; turbo ion-spray gas flow, 50 psig; curtain gas flow, 20 psig; collision gas (nitrogen) pressure, 5 Torr; declustering potential, 71 eV; and entrance potential, 10 eV. The mass transitions for sauchinone and the IS were *m/z* 357.15 → 327.40 and 397.09 → 341.20, respectively, at a collision energy of 17 eV and a collision cell exit potential of 8 eV, respectively. Quadrupoles Q1 and Q3 were set to unit resolution. The analytical data were processed using Analyst software (Version 1.5.1; Applied Biosystems).

Method validation assays were carried out according to the currently accepted United States Food and Drug Administration's bioanalytical method validation procedure.[22] The validation parameters were selectivity, linearity, sensitivity, precision, accuracy, matrix effect, and stability of sauchinone in mouse plasma.

To prepare the standard and quality control (QC) samples of sauchinone, stock solutions of sauchinone were serially diluted with methanol and added to drug-free plasma to obtain final concentrations of 1, 5, 10, 50, 500, and 5000 ng/mL. A 100 μL aliquot of acetonitrile containing 0.5 μg/mL IS was added to a 50 μL aliquot of mouse plasma and mixed. After vortex-mixing and centrifugation (15000×***g*** for 10 min), 20 μL of the supernatant was directly injected into the column. On the day of analysis, calibration curves for sauchinone in mouse plasma were derived from their peak area ratios relative to that of the IS using linear regression with $1/x$ as the weighting factor. The concentrations of 5, 500, and 5000 ng/mL for sauchinone were used for QC samples.

Selectivity was evaluated by comparing chromatograms of six different batches of plasma obtained from six subjects to ensure that no interfering peaks were present at the respective retention times of the analytes at the lower limit of quantitation (LLOQ) level. The LLOQ was defined as the lowest concentration of analyte yielding a signal-to-noise ratio of at least 10, acceptable accuracy (80–120%), and sufficient precision (within 20%); LLOQs were verified by analyzing five replicates. Also the concentrations under the LLOQ were regarded as the zero value and discarded as mentioned in FDA guideline (estimation of concentrations below LLOQ is not recommended).[22]

The linearity of each method-matched calibration curve was determined by $y=ax+b$ by plotting the peak area ratios (y) of sauchinone relative to that of the IS *vs.* the nominal concentration (x) of the same analyte. The calibration curves were constructed with a weighting factor and a mean linear regression equation.

Intra- and inter-day accuracy and precision were determined at six different concentrations on five consecutive days; on each day, six replicates were analyzed with independently prepared calibration curves. The percentage accuracy was expressed as {(mean observed concentration)/(nominal concentration)}×100, and the precision was the relative standard deviation (RSD, %). All assays were performed in triplicate at concentrations of 1, 5, 10, 50, 500, and 5000 ng/mL sauchinone.

To evaluate the matrix effect and stability of sauchinone in mouse plasma, drug-free plasma samples were spiked with 10, 500, and 5000 ng/mL sauchinone. The matrix effect was calculated by taking the analyte peak areas obtained by direct injection of solvent (or neat) standard solutions as A, those for solvent (or neat) standard solutions spiked after sample preparation as B.[23]

$$\text{Matrix effect (\%)} = 100 \times \text{B/A}$$

The matrix effect of the IS was evaluated using the same method. Stability was assessed by analyzing three replicate samples after five different manipulations: (1) short-term storage (24 h at room temperature); (2) long-term storage (21 d at −20°C); (3) three freeze-thaw cycles; and (4) post-treatment storage (12 h at room temperature). The concentrations obtained were compared with nominal values of the prepared

samples.

On the start of the each analysis for all experiments, the calibration curve was plotted based on the above method. Also the QC samples were assayed with each batch of plasma samples to consider the analytical conditions are appropriated within the run and daily QC samples were implemented for HPLC-MS/MS assay.

Intravenous and Oral Studies The surgical procedures including the cannulation of the carotid artery and the jugular vein were conducted under intramuscular injection anesthesia with a 125 mg (1.5 mL)/kg tiletamine HCl and zolazepam HCl mixture. After mice were awakened from anesthesia, the administration of sauchinone and the sequential blood sampling from the carotid artery were started.

Sauchinone (dissolved in PEG 400 : distilled water=9 : 1, v/v) at doses of 7.5, 20, and 50 mg (5 mL)/kg was intravenously administered through the jugular vein (n=7, 8 and 7, respectively). The microsampling system was programmed to collect a 10 μL blood sample into a micro-vial containing 50 μL of 12.5 units/mL heparinized saline. Blood loss due to blood sampling was replaced with equal volumes of heparinized saline. Samples were collected at 0, 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, or 540 min after intravenous administration of sauchinone with virtually no blood loss. After centrifugation of each micro-vial, a 50 μL of supernatant was collected. At the end of 24 h, each metabolic cage was rinsed with 5 mL distilled water and the rinse was combined with the 24 h urine sample in the urine collector. At the same time (24 h), each mouse was sacrificed by cervical dislocation, and the entire gastrointestinal tract (GI; including its contents and feces) was removed, transferred to a beaker containing 10 mL of methanol (to facilitate extraction of sauchinone) and the GI tract was cut into small pieces using scissors. After manual shaking and stirring, a 50 μL aliquot of the supernatant was collected from each beaker and stored.

Sauchinone (the same solution as used in the intravenous study) at doses of 20, 100, and 500 mg (10 mL)/kg was administered orally using a gastric gavage tube in mice after overnight fasting with free access to water (n=7, 8 and 7, respectively). A blood sample was collected *via* the carotid artery at 0, 5, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, or 540 min after the oral administration. Other procedures were similar to those in the intravenous study. All biological samples were stored at −80°C until we determined sauchinone concentrations by HPLC-MS/MS.

Tissue Distribution of Sauchinone after Intravenous and Oral Administration The surgical procedures and administration of sauchinone were conducted the same as the intravenous and oral pharmacokinetic studies as mentioned above and using a method reported previously.[24] At 30, 120, and 360 min after intravenous and oral administration of sauchinone at doses of 20 and 100 mg/kg, respectively, as much blood as possible was collected *via* the carotid artery, and each mouse was sacrificed by cervical dislocation. Approximately 1 g of each liver, stomach, small intestine, large intestine, kidney, heart, lung, spleen, muscle, fat, mesentery and brain was excised and homogenized with four-fold volume of distilled water. After centrifugation, the supernatant was collected and all samples were stored at −80°C until the determining the concentrations of sauchinone by HPLC-MS/MS.

In Vitro* Metabolism of Sauchinone in S9 Fractions of Tissues** The procedures using tissue homogenates were similar to a method reported previously.[24,25] Approximately 1 g of each liver, stomach, small intestine, large intestine, kidney, fat, muscle, heart and brain was excised after cervical dislocation (n=5). Each tissue sample was rinsed with cold 0.9% NaCl-injectable solution, blotted dry with tissue paper, and weighed. Each tissue sample was homogenated with four-volume of 0.25 M sucrose. Metabolic activity was initiated by adding a 130 μL aliquot of the 9000×g*** supernatant fraction of each tissue to a 100 μL aliquot of Tris-buffer (pH 7.4), a 5 μL of 0.9% NaCl-injectable solution containing 1 μg/mL sauchinone, a 5 μL (1 mM) aliquot of NADPH, a 5 μL (3.3 mM) aliquot of UDPGA, and a 5 μL (2 mM) aliquot of PAPS. A 250 μL aliquot of acetonitrile (containing 500 μg/mL of the IS) was added after 30 min incubation in a thermomixer (37°C and 500 rpm; Eppendorf, Hamburg, Germany) to terminate enzyme activity. The amount of remaining sauchinone in each tissue was determined by HPLC-MS/MS analysis.

Tentative Metabolites of Sauchinone Identified from MS and MS/MS Data in *in Vivo and in Vitro* Systems Based on the *in vitro* metabolism studies as above mentioned, the metabolites of sauchinone were estimated from MS and MS/MS data using the modified analytical method with gradient mobile phase and HPLC-MS/MS methods. The mass transitions for sauchinone were m/z 357.15→327.40 (collision energy of 19 eV and collision cell exit potential of 12 eV, respectively), 357.15→345.20 (13 and 12 eV, respectively), and 357.15→257.10 (33 eV and 12 eV, respectively). The mobile phase composition was started with 95 : 5 (v/v) of distilled water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B), followed by a linear increase to 5 : 95 (v/v) of A : B which was achieved at 15 min. The gradient was returned to 95 : 5 (v/v) of A : B. The MRM methods included the possible metabolites by phase I and/or II reactions as followings: oxidation, (m/z 373.15→327.40, 373.15→343.40, or 373.15→273.10), dioxidation (m/z 389.14→327.40), methylation (m/z 371.17→327.40), demethylation (m/z 343.13→257.10), dehydrogenation (m/z 355.13→255.08 or 355.13→257.10), and bis-glucuronide conjugation (m/z 709.22→609.17). Also these metabolites were predicted in plasma samples after oral administration using this modified HPLC-MS/MS methods.

Mouse Plasma Protein Binding of Sauchinone Using the Equilibrium Dialysis Technique A 250 μL aliquot of mice plasma was dialyzed against 250 μL of isotonic Sørensen phosphate buffer (pH 7.4) containing 3% (w/v) dextran (buffer) in a dialysis cell using a Spectra/Por 4 membrane (molecular weight cutoff of 12000–14000; Spectrum Medical Industries, Sanford, ME, U.S.A.). Sauchinone were spiked into the plasma compartment to produce an initial concentration of 1 μg/mL and other procedures followed a method reported previously.[26]

Pharmacokinetic Analysis Standard methods[27] were used to calculate the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin 2.1; Pharmasight Corp., Mountain View, CA, U.S.A.): the total area under the plasma concentration–time curve from time zero to infinity (AUC), time-averaged total body, renal, and non-renal clearances (CL, C_R, and CL_{NR}, respectively), terminal half-life, mean residence time (MRT), and apparent volume of distribution at steady state (V_{ss}). The extent of absolute oral bioavailability (F) was calculated by dividing the AUC_{oral}/AUC_{iv}. The

peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the extrapolated data.

Statistical Analysis A p-value <0.05 was deemed to be statistically significant using a student t-test between the two means for the unpaired data or a Duncan's multiple range test of Social Package of Statistical Sciences (SPSS) *posteriori* ANOVA among the three means for the unpaired data. All data are expressed as mean±standard deviations except median (ranges) for T_{max}.

RESULTS

All analytes displayed protonated molecular ions $([M+H])^+$ in positive ion mode as the major species. The fragmentation patterns of the protonated molecular ions were evaluated by increasing collision energy. The structures and ion spectra for sauchinone and the IS are shown in Fig. 1; the greatest intensities were observed at m/z 327.40 and 341.20, respectively. The mass parameters were optimized by observing the maximal response of the product ions.

The mass spectrometry and chromatographic conditions, particularly the composition of the mobile phase, were optimized to achieve good resolution and symmetrical peak shapes for sauchinone and the IS, with acceptable retention factors ($k' \geq 2$) and a short run time. Sauchinone and the IS eluted at 2.5 and 5.0 min, respectively.

No interfering peaks were detected at these elution times. Typical chromatograms for drug-free plasma, plasma spiked with 50 ng/mL sauchinone, and plasma collected from mice 540 min after oral administration of 100 mg/kg sauchinone are shown in Fig. 2. The total run time per sample was 6.5 min.

The calibration curves for mouse plasma provided reliable responses at sauchinone concentrations of 1–5000 ng/mL, respectively. The best linear fit and least-squares residuals for the calibration curve were achieved with a weighting factor of $1/x$. During the validation, the mean correlation coefficients (r) in mouse plasma were 0.999 for sauchinone. The LLOQ for sauchinone was 1 ng/mL at a signal-to-noise ratio of 10. This sensitivity was sufficient to allow pharmacokinetic studies of sauchinone after intravenous and oral administration. The intra- and inter-day precision and accuracy of the assay were determined by analyzing five replicates of standard samples at six concentrations on five consecutive days (Table 1). The coefficients of variation for the intra- and inter-day precision of sauchinone were <10.0% and <6.68%, respectively. The intra- and inter-day accuracies of sauchinone were 96.2–107% and 99.6–114%, respectively. The matrix effect of sauchinone was 83.5–112%, indicating that the impact from the prepared plasma matrix was negligible and consistent. No significant degradation of any of the analytes in mouse plasma occurred after short-term storage for 24 h at room temperature, long-term storage for 21 d at –20°C, three freeze-thaw cycles, or post-treatment storage for 12 h at room temperature, with ±15% deviation between the predicted and nominal concentrations (Table 2).

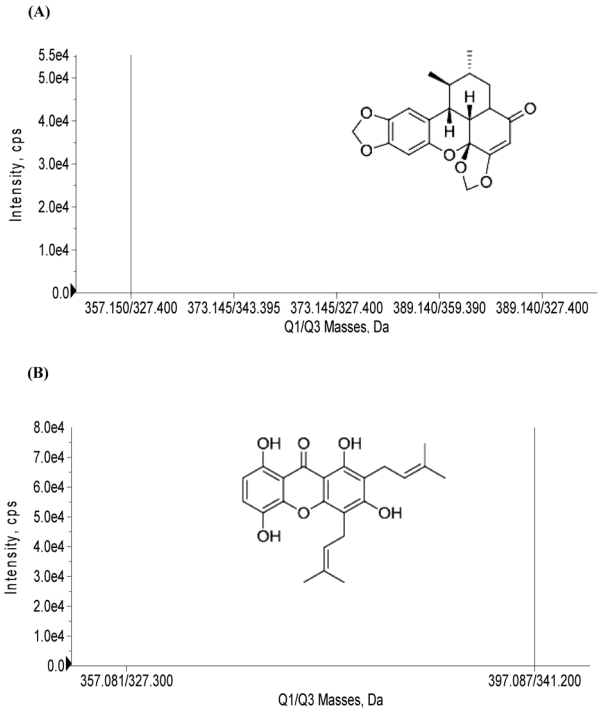

Fig. 1. The Structures and Ion Spectra for Sauchinone (A) and Gartanin (IS; B)

The HPLC-MS/MS method described herein was successfully applied to a pharmacokinetic study of sauchinone. The mean arterial plasma concentration–time profiles of sauchinone after intravenous and oral administration of sauchinone to mice are shown in Fig. 3 and the relevant pharmacokinetic parameters are listed in Table 3. After intravenous administration of sauchinone at doses of 7.5 and 20 mg/kg, the *AUC*s of sauchinone were dose-proportional and other pharmacokinetic parameters were comparable. At 50 mg/kg, the normalized *AUC* was significantly greater and CL (and CL_{NR}) values were significantly slower than those at 7.5 and 20 mg/kg, respectively. After oral administration at doses of 20, 100, and 500 mg/kg, the absorption of sauchinone from the gastrointestinal tract was rapid; sauchinone was detected in the plasma from the first or second blood sampling time (5 or 15 min), and rapidly reached T_{max} (5–15 min) for three doses studied. The normalized *AUC*s and other pharmacokinetic parameters were not significantly different among three doses studied. The F value was calculated at 20 mg/kg.

In tissue distribution studies, the amount of sauchinone recovered from tissues at 30, 120, and 360 min after intravenous and oral administration to mice are listed in Table 4. In the intravenous study, tissue/plasma (T/P) ratios of sauchinone were less than unity (T/P=1) for most tissues except liver (at 30, 120, and 360 min), small intestine (at 30 min), and kidney (at 120 and 360 min), lung, muscle, fat and mesentery (at 360 min). In the oral study, the T/P ratios of sauchinone were greater than unity in the liver, stomach, small intestine, large intestine (at 30, 120, and 360 min), kidney, fat, and mesentery (at 30 and 120 min) and lung (at 30 min) and their T/P values became increased as time goes on. Moreover, the almost T/P ratios of sauchinone in oral study were greater than those in intravenous study at same tissues, suggesting that the distribution of sauchinone to tissues after oral administration is greater than those after intravenous administration.

In the *in vitro* metabolism studies using 9000×***g*** supernatant (S9) fractions of tissues, the values for the disappearance of sauchinone after 30-min incubating sauchinone are listed in Table 5. Mouse liver and small intestine showed some metabolic activities of sauchinone.

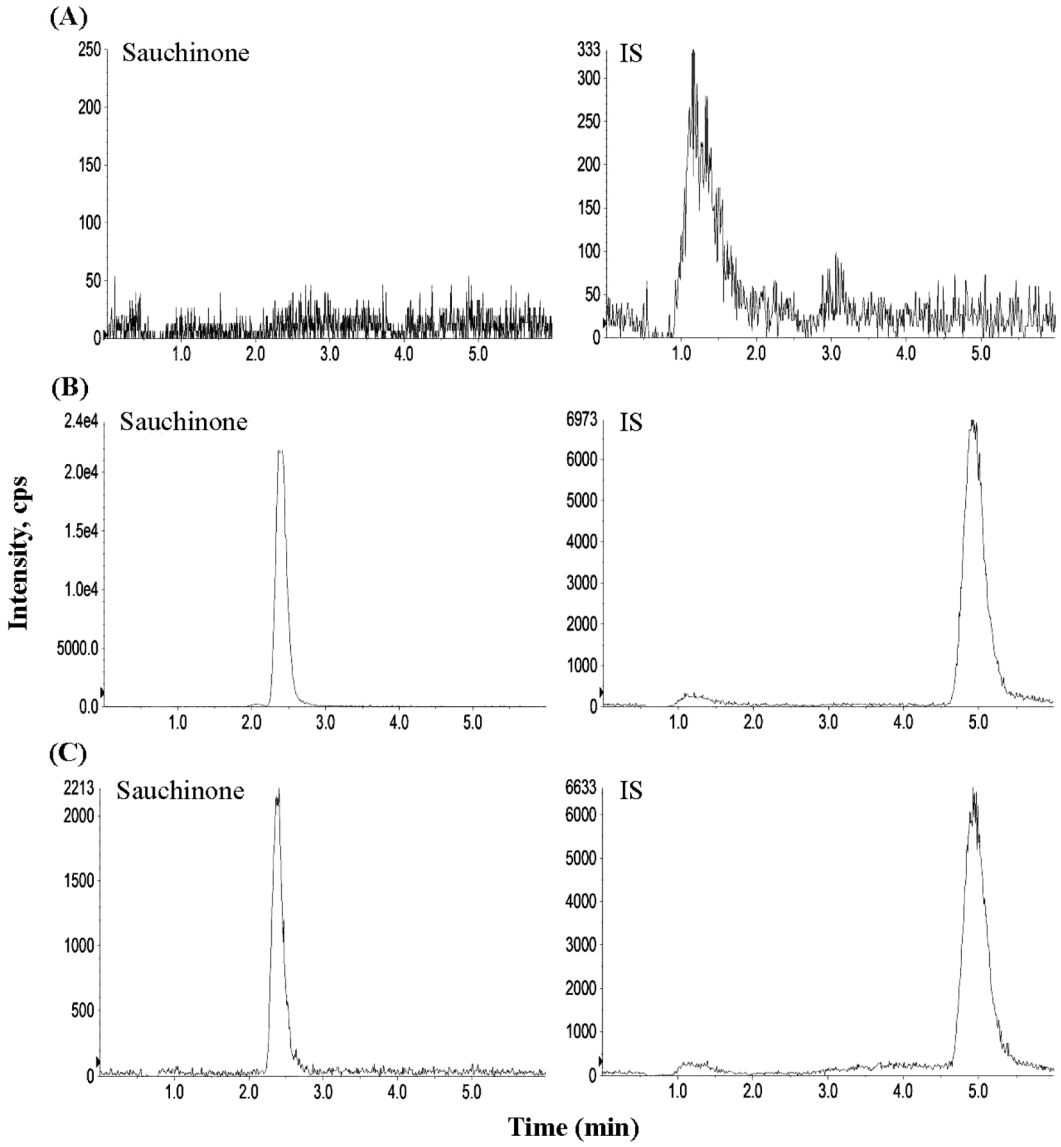

Fig. 2. Mass Chromatogram after Deproteinization with Acetonitrile for the Mouse Blank Plasma (A), the Mouse Plasma Spiked with 50 ng/mL Sauchinone (B), and Mouse Plasma Sample at 540 min after Oral Administration at a Dose of 100 mg/kg (C)

Table 1. Intra- and Inter-Day Precision and Accuracy for Identifying Sauchinone in Mouse Plasma

Theoretical concentration (ng/mL)	Precision		Accuracy (%)
	Mean±S.D.	RSD[a)] (%)	
Intra-day			
1	0.962±0.0758	7.88	96.2
5	5.07±0.509	10.0	101
10	9.93±0.586	5.91	99.3
50	53.5±4.18	7.81	107
500	496±40.7	8.22	99.2
5000	4997±161	3.23	99.9
Inter-day			
1	1.14±0.0672	5.88	114
5	5.14±0.343	6.68	103
10	10±0.499	4.98	100
50	51.9±2.2	4.24	104
500	498±30.2	6.05	99.6
5000	5004±155	3.10	100

a) RSD, relative standard variation (S.D.×100/mean).

The observed tentative metabolites in plasma or S9 fractions of tissues from MS and MS/MS data were as the following (Fig. 4): metabolites by methylation (*m/z* 371.17; gain 14.02 Da), oxidation (*m/z* 373.15; gain 16.00 Da), and dioxidation (*m/z* 389.14; gain 31.99 Da) in plasma after oral administration; oxidation (*m/z* 373.15; gain 16.00 Da) in S9 fraction of liver; dehydrogenation (*m/z* 355.13; loss 2.02 Da), oxidation (*m/z* 373.15; gain 16.00 Da) in S9 fraction of small intestine; dehydrogenation (*m/z* 355.13; loss 2.02 Da), oxidation (*m/z* 373.15; gain 16.00 Da) and bis-glucuronide conjugation (*m/z* 709.22; gain 352.07 Da) in S9 fraction of large intestine; and demethylation (*m/z* 343.13; loss of 14.02 Da), dehydrogenation (*m/z* 355.13; loss 2.02 Da), and oxidation (*m/z* 373.15; gain 16.00 Da) in S9 fraction of kidney.

The protein binding value of sauchinone to fresh mouse plasma at a concentration of 1 μg/mL was 53.0±11.2% using the equilibrium dialysis technique.

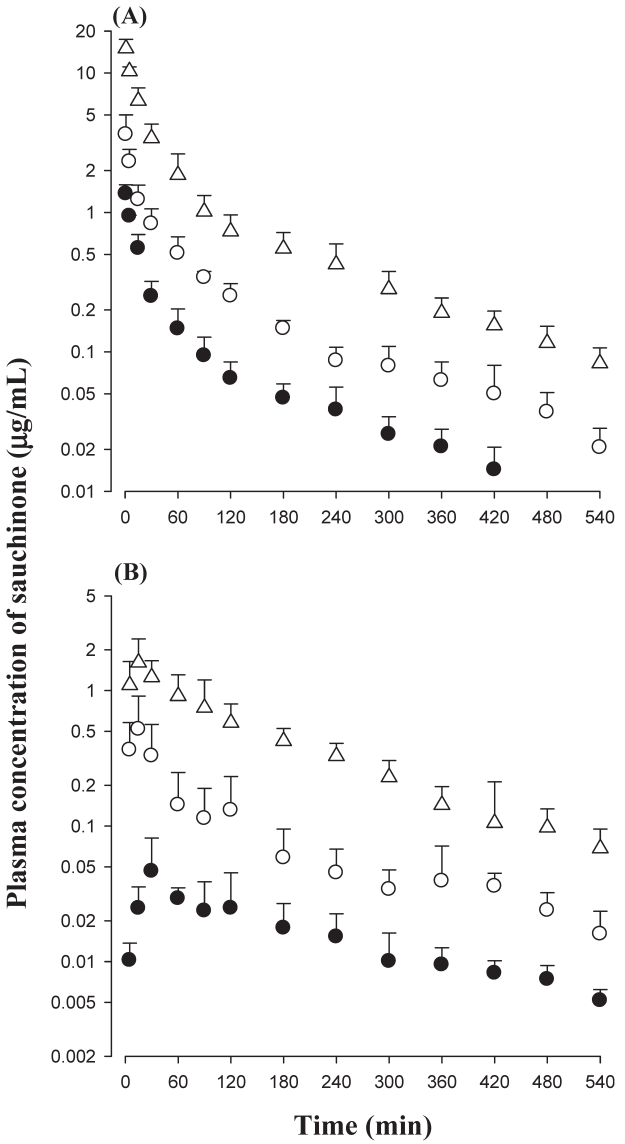

Fig. 3. Mean Arterial Plasma Concentration–Time Profile of Sauchinone after Intravenous Administration of Sauchinone at Doses of 7.5 (n=7; ●), 20 (n=8; ○) and 50 (n=7; △) mg/kg, Respectively, to Mice (A) Also Those of Sauchinone after Oral Administration of Sauchinone at Doses of 20 (n=7; ●), 100 (n=8; ○) and 500 (n=7; △) mg/kg, Respectively, to Mice (B)

Bars represent standard deviation.

DISCUSSION

The full scan positive mass spectra of sauchinone and the IS exhibited deprotonated mass ions in the Q1 spectrum. These ions were used as precursors to obtain product ion spectra and were well resolved in the analysis of sauchinone in animal plasma. In both the analytical procedures, the inter- and intra-day precisions were <15%, and accuracy was 80–120% (Table 1), which complies with FDA regulations.[22] The LC-MS/MS analytical method improved the detection limit, and the microsampling system, which obtained a 10 μL sample at each sampling point, enabled us to carry out a pharmacokinetic evaluation in mice. Moreover, the microsampling system minimized catheter occlusion due to blood clots, blood loss and immobilization stress caused by manual blood sampling such as heart puncture, which led to minimal blood loss and stress when collecting multiple blood samples collection from a single animal.[28]

In intravenous studies, there was no difference in the normalized *AUC*s and other pharmacokinetic parameters between 7.5 and 20 mg/kg, indicating that pharmacokinetics of sauchinone from 7.5 to 20 mg/kg were in the linear ranges in mice. In contrast to low doses (7.5 and 20 mg/kg), the normalized *AUC* was significantly greater and the CL (and CL_{NR}) was significantly slower, respectively, at 50 mg/kg. Thus, it is evident that sauchinone possessed the nonlinear pharmacokinetic properties at intravenous 50 mg/kg in mice. The contribution of gastrointestinal excretion of unchanged sauchinone to its CL_{NR} was almost negligible; the percentage of the dose recovered from the entire gastrointestinal tract (including its contents and feces) at 24 h (GI_{24h}) was only 0.0906% of the intravenous dose (Table 3). However, the low GI_{24h} of 0.0906% was not likely due to chemical and enzymatic degradation of

Table 2. Matrix Effect and Stability of Sauchinone

	10 (ng/mL)		500 (ng/mL)		5000 (ng/mL)	
	Accuracy (%)	RSD[a] (%)	Accuracy (%)	RSD[a] (%)	Accuracy (%)	RSD[a] (%)
Matrix effect (%)	83.5	10.1	112	5.04	95.8	3.64
Stability						
Room temperature/24 h	108	4.94	99.4	5.68	100	9.16
–20°C/21 d	109	3.90	98.2	3.89	96.3	7.41
–20°C/3 freeze–thaw cycles	102	12.9	100	9.89	100	5.90
Room temperature/12 h (deproteinization solvent)	103	6.85	99.8	9.69	108	3.98

a) RSD, relative standard variation (S.D.×100/mean).

Table 3. Mean (± Standard Deviation) Pharmacokinetic Parameters of Sauchinone after Intravenous (7.5, 20 and 50 mg/kg, Respectively) and Oral (20, 100, and 500 mg/kg, Respectively) Administration to Mice

Parameter	Doses		
Intravenous	7.5 mg/kg (n=7)	20 mg/kg (n=8)	50 mg/kg (n=7)
Body weight (g)	28.0±1.15	27.4±0.818	27.5±2.08
AUC (μg min/mL)[a]	44.7±9.23	126±20.5	498±113
Terminal half-life (min)	151±17.1	126±7.13	141±12.7
MRT (min)	122±27.5	129±9.80	118±19.6
V_{SS} (mL/kg)	5617±829	5136±1074	4637±872
CL (mL/min/kg)[b]	144±13.7	168±29.7	97.8±29.3
CL_R (mL/min/kg)	0.0773±0.0431	0.0514±0.0212	0.0462±0.0171
CL_{NR} (mL/min/kg)[b]	144±13.6	168±29.7	97.8±29.3
Ae_{0-24h} (% of sauchinone dose)	0.0521±0.0241	0.0319±0.0165	0.0474±0.0231
GI_{24h} (% of sauchinone dose)	0.0746±0.0284	0.0906±0.0511	0.0510±0.0358
Oral	20 mg/kg (n=7)	100 mg/kg (n=8)	500 mg/kg (n=7)
Body weight (g)	26.7±3.92	23.2±5.17	24.5±2.13
AUC (μg min/mL)	9.78±2.18	43.6±20.8	229±74.3
Terminal half-life (min)	228±14.9	175±63.7	151±47.1
C_{max} (μg/mL)	0.0566±0.0266	0.543±0.380	1.77±0.811
CL_R (mL/min/kg)	0.0309±0.0102	0.0235±0.0158	0.0309±0.0175
T_{max} (min)[c]	15 (15–120)	5 (5–30)	15 (15–30)
GI_{24h} (% of sauchinone dose)	22.1±9.72	24.8±14.6	21.8±6.85
F (%)	7.76	—	—

a) Normalized AUC of 50 mg/kg was significantly different from those of 7.5 and 20 mg/kg by ANOVA test. *b*) The pharmacokinetic parameter at 50 mg/kg was significantly different from those of 7.5 and 20 mg/kg by ANOVA test. *c*) Median (ranges).

Table 4. Mean (± Standard Deviation) Amount (ng/mL for Plasma and ng/g for Other Tissues) of Sauchinone Recovered from Tissues at 30, 120, and 360 min after Intravenous and Oral Administration at Doses of 20 and 100 mg/kg, Respectively, to Mice

Tissue	Intravenous			Oral		
	30 min (n=6)	120 min (n=6)	360 min (n=6)	30 min (n=6)	120 min (n=6)	360 min (n=6)
Plasma	588±13.1	173±28.4	46.6±8.13	112±4.18	81.2±10.5	58.9±22.2
Liver	1544±362	446±143	47.1±12.3	4700±578	6960±350	112±52.5
	(2.63)[a]	(2.58)	(1.01)	(42.1)	(85.7)	(1.90)
Stomach	132±19.4	84.2±17.8	39.4±25.1	9330±1410	10300±394	109±58.1
	(0.224)	(0.486)	(0.845)	(83.6)	(127)	(1.85)
Small intestine	1034±301	156±36.7	19.4±5.31	3660±1520	4760±733	188±105
	(1.76)	(0.902)	(0.417)	(32.8)	(58.6)	(3.19)
Large intestine	204±27.1	104±25.0	26.4±5.82	300±282	5550±2800	323±99.1
	(0.347)	(0.603)	(0.558)	(2.69)	(68.4)	(5.48)
Kidney	466±109	668±118	101±7.13	544±261	592±111	19.9±21.5
	(0.793)	(3.86)	(2.18)	(4.88)	(7.29)	(0.338)
Heart	148±22.6	35.4±3.32	42.8±13.7	51.4±9.12	78.3±22.6	24.2±6.64
	(0.252)	(0.204)	(0.918)	(0.461)	(0.961)	(0.411)
Lung	202±47.3	46.2±25.6	53.2±2.98	163±34.6	79.2±13.5	24.2±6.67
	(0.344)	(0.267)	(1.14)	(1.46)	(0.975)	(0.411)
Spleen	133±11.7	51.8±18.4	43.2±14.4	40.6±8.91	62.6±18.5	37.4±29.8
	(0.226)	(0.299)	(0.927)	(0.358)	(0.771)	(0.635)
Muscle	43.8±7.72	16.1±2.34	79.2±10.3	13.5±4.80	26.6±5.09	28.5±15.8
	(0.0745)	(0.0927)	(1.70)	(0.121)	(0.328)	(0.484)
Fat	40.2±5.41	117±32.2	102±25.5	183±28.3	504±156	31.3±19.2
	(0.0684)	(0.678)	(2.19)	(1.64)	(6.21)	(0.531)
Mesentery	105±32.4	50.4±7.74	119±13.7	122±57.1	240±113	49.2±21.5
	(0.179)	(0.291)	(2.56)	(1.10)	(2.96)	(0.835)
Brain	96.5±23.7	7.86±0.943	28.4±13.1	9.56±1.61	19.1±4.13	8.61±5.03
	(0.164)	(0.0454)	(0.609)	(0.0857)	(0.235)	(0.146)

a) Values in parentheses are values of the tissue-to-plasma (T/P) ratio.

Table 5. Mean (±Standard Deviation) Values for the Disappearance of Sauchinone after 30-min Incubation of 1 μg/mL Sauchinone in S9 Fractions of Various Mouse Tissues

Tissue	Disappearance of sauchinone (% of the spiked sauchinone)
Liver	24.7±2.62
Stomach	4.62±4.45
Small intestine	30.5±7.51
Large intestine	3.55±5.73
Kidney	5.17±1.72
Fat	8.20±3.98
Muscle	12.1±3.39
Heart	8.20±1.92
Brain	2.83±3.98

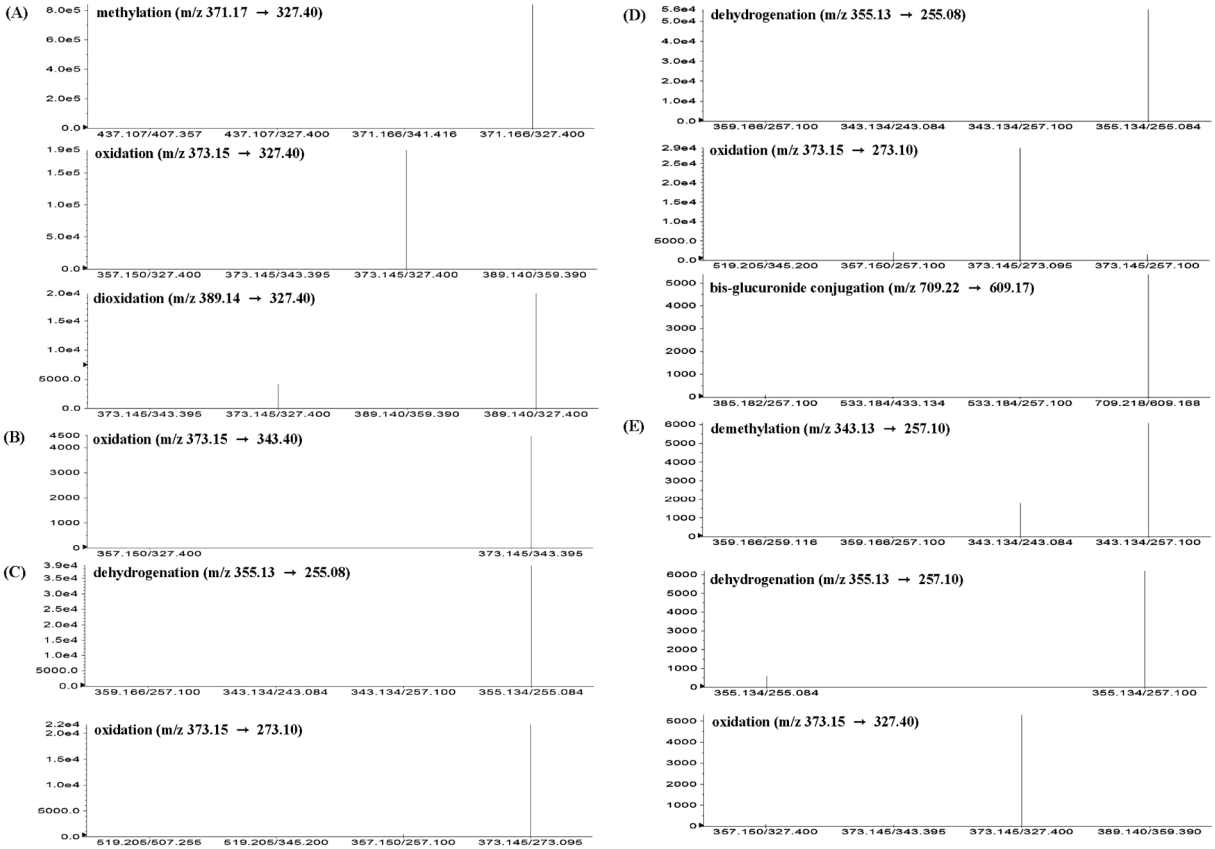

Fig. 4. MS/MS Spectra of Metabolites in Plasma after Oral Administration (A), S9 Fractions of Liver (B), Small Intestine (C), Large Intestine (D), and Kidney (E)

sauchinone in gastric fluid, as sauchinone was stable for up to 24 h in various buffer solutions with pHs of 3–10 and in GI samples from mice (at least 93.1% of spiked sauchinone remains in all samples; our unpublished data). Thus, the CL_{NR} of sauchinone could represent its metabolic clearance. Moreover, the ratio of CL_{NR}/CL of almost 1 explained that the metabolic pathway is a main route in the elimination of sauchinone. Thus, the metabolism of sauchinone was saturated at 50 mg/kg, which made for the nonlinear pharmacokinetics of sauchinone.

The CL of sauchinone (168 mL/min/kg; Table 3) was slower than the cardiac output (8 mL/min/0.02 kg) and faster than hepatic blood flow rate (1.8 mL/min/0.02 kg) based on the plasma data (using a hematocrit of 0.45 in mice).[13,14] These data indicate that sauchinone was metabolized in systemic first-pass effects *via* hepatic and extra-hepatic organs. This was demonstrated by *in vitro* metabolism studies using tissue homogenates; 24.7 and 30.5% of spiked sauchinone disappeared (mainly metabolized after a 30 min incubation of 1 μg/mL sauchinone with S9 fractions of the liver and small intestine, respectively; Table 5). These results suggest that sauchinone was metabolized *via* the liver and small intestine.

The CL_Rs of sauchinone were estimated based on free (unbound to plasma proteins) fractions (47.0%) in plasma ($CL_{R,fu}$); the $CL_{R,fu}$ value thus estimated was 0.109 mL/min/kg. This value was considerably slower than the glomerular filtration rate of 14.0 mL/min/kg in mice (based on creatinine clearance),[13,14] suggesting that sauchinone is excreted into urine predominantly *via* glomerular filtration in mice.

In oral studies, all doses of sauchinone at 20, 100, and 500 mg/kg were included in the linear pharmacokinetic ranges. The *F* value of sauchinone was only 7.76% (Table

3), which was calculated based on the linear pharmacokinetics of sauchinone at 20 mg/kg. To ascertain whether the poor gastrointestinal absorption of sauchinone caused the low F, the "true" fraction of the oral dose of unabsorbed sauchinone (F_{unabs}) was calculated from the intravenous GI_{24h}, F and oral GI_{24h} values.[29] The F_{unabs} value estimated was 0.221. Thus, the absorbed fraction of sauchinone was 0.779. These data indicate that the low F of sauchinone was caused by the low gastrointestinal absorption and other reasons which could be due to considerable hepatic and intestinal metabolism as mentioned above. Metabolism of sauchinone in the liver and small intestine was supported by the *in vitro* metabolism study.

Probable metabolites of sauchinone were detected by oxidation (*m/z* 373.15→327.40, 373.15→343.40, or 373.15→273.10), dioxidation (*m/z* 389.14→327.40), methylation (*m/z* 371.17→327.40), demethylation (*m/z* 343.13→257.10), dehydrogenation (*m/z* 355.13→255.08 or 355.13→257.10), or bis-glucuronide conjugation (*m/z* 709.22→609.17) in plasma or S9 fractions of liver, small intestine, large intestine or kidney at this time. Because the MS/MS fragments of sauchinone were 327.40, 345.20, and 257.10, the mass transition of daughter ion in oxidated sauchinone were 327.40, 343.40, and 273.10. These observed metabolites suggested that sauchinone were metabolized *via* phase I/II reactions and circulated into the blood.

The T/P ratios in the liver, stomach, small intestine, large intestine, lung, muscle, fat, and/or mesentery were greater than unity, suggesting that sauchinone was distributed in various tissues with high affinity and might have efficacy or toxicity in these target tissues regardless of its plasma concentration. Although the plasma concentration of sauchinone was low after oral administration or at later sampling points, the pharmacological activities of sauchinone could be existing due to the higher T/P ratios in targeted tissues. Also in another aspects, the T/P ratios becomes higher in several tissues as times go on even at 360 min with decreased concentrations of sauchinone, suggesting that high concentrations of sauchinone in tissue might be somewhat related with low concentration in the plasma. Of course, the T/P ratios of sauchinone in various tissues were different depending on the administration route of sauchinone and sampling time points. In aspects of mechanism of action, it was reported that the phosphorylated AMPK levels in the liver was induced by sauchinone after oral administration.[17] Considering the low F value of sauchinone, the high T/P ratio of sauchinone in the liver seemed to contribute the phosphorylated AMPK levels in the liver, which might bring about the capacity of sauchinone for the protection the hepatocytes from oxidative stress induced by fat accumulation after oral administration. This relationship between T/P ratios and pharmacological activity at specific tissues could provide clues for the efficacy of herbal products based on the pharmacokinetic characteristics.

In conclusion, the HPLC-MS/MS and microsampling system was a useful drug development tool that provided reliable and accurate pharmacokinetic parameters from small numbers of mice by minimizing the shortcomings of manual technique (*e.g.*, inter-animal and physiological variations). As a result, the F of sauchinone was low at 7.76%, and this could be due to considerable hepatic and/or intestinal metabolism of sauchinone in mice. Also the probable metabolites by oxidation, dioxidation, methylation, demethylation, dehydrogenation or bis-glucuronidation were detected in plasma, S9 fractions of liver or intestine at this time. The distribution and affinities of sauchinone were considerable particularly in mice's the liver, stomach, small intestine, large intestine, kidney, lung, muscle, fat and/or mesentery although they could be changeable depending on the administration route or sampling time. These pharmacokinetic data of sauchinone provide an important basis for preclinical applications. Furthermore, these experimental methods can be adjusted to evaluate the pharmacokinetics of other natural products in mice.

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Conflict of Interest The authors declare no conflict of interest.

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