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A new approach for pharmacokinetic studies of natural products: measurement of isoliquiritigenin levels in mice plasma, urine and feces using modified automated dosing/blood sampling system

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ABSTRACT: The present study was undertaken to investigate the pharmacokinetics of isoliquiritigenin (isoLQ) as determined by the automated dosing/blood sampling (ABS) and traditional manual blood sampling techniques in awake and freely moving mice using combined liquid chromatography tandem mass spectrometry. Pharmacokinetic comparison was conducted by allocating mice into two groups; an ABS group (intravenous study and oral studies, $n = 5$ each) and a manual group (intravenous and oral studies; $n = 5$ each). Significant differences in pharmacokinetic parameters (area under the curve and clearances) were observed between ABS and manual groups. This could be mainly due to the blood sampling site difference (via heart puncture in traditional manual group and via carotid artery in ABS groups). The low *F* of isoLQ could be mainly due to a considerable gastrointestinal and/or hepatic first-pass effect and not to incomplete absorption. The driving force for distribution and elimination of drugs is its concentration in the arterial blood. Therefore, the ABS method was found to be a useful drug development tool for accelerating the process of preclinical *in vivo* studies and for obtaining reliable and accurate pharmacokinetic parameters in mice.

Keywords: automated blood sampling; isoLQ; mouse; LC-MS/MS; pharmacokinetics

Introduction

Pharmacokinetic studies to investigate the absorption, distribution, metabolism and excretion of pharmacologically active compounds of interest are routine, but essential steps both in preclinical and clinical studies. Since therapeutic outcome depends on the toxicity and safety of compounds in the body, the pharmacokinetic parameters, which are calculated from plasma concentrations of compounds as a function of time and represent the effect on the body of the compounds, are fundamental (De Smet and Brauwers, 1997). In particular, the importance of pharmacokinetic studies in herbal components is increasing because a large number of herbal products are used as adjuvant or alternative medicines based on almost *in vitro* pharmacodynamic studies and, moreover, natural products have been known to synergize or antagonize drug effects (Esimone *et al*., 2002; Singh, 2006). Nevertheless, the pharmacokinetic approach for natural products, usually comprising a mixture of known and/or unknown components and a single component, has been challenged because of the complexity of natural products, the unavailability or inadequacy of reference standards and the presence of diverse sample preparations for even single plants, unlike pharmaceuticals (Hussain *et al*., 2011). Albeit limited, there have been several pharmacokinetic studies for natural products, such as artemisinin, berberine and galanthamine (Goh *et al*., 2011; Medhi *et al*., 2009; Deng *et al*., 2008). From these pioneering pharmacokinetic works for natural products as well as other drugs, it is demonstrated that pharmacokinetic tools can be applied to natural products to produce more profiles, as requested for evidence-based drugs. In this regard, pharmacokinetic investigation of natural products may facilitate the use of natural products as adjuvant or alternative medicines. Hence, it is necessary to establish the general pharmacokinetic experimental tools of natural products at preclinical levels (Mills and Bone, 2000).

The common pharmacokinetic studies using a manual serial blood sampling technique show a good correlation for pharmacokinetic aspects (e.g. absorption and metabolism) in small animals (e.g. rats) and humans (Chiou and Barve, 1998). However, in mice, relatively small amounts of natural products in pharmacokinetic studies are inevitably used, and this makes them an effective choice instead of rats, since it is arduous to secure sufficient amounts of natural compounds. Also the frequency and necessity of the use of various knockout or xenografted mouse models to prove the pharmacological effects of natural compounds is increasing in preclinical investigations.

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Abbreviations used: ABS, automated dosing/blood sampling; isoLQ, isoliquiritigenin.

Because a sufficient plasma volume is required for analysis, only one blood sample was obtained from one mouse and this methodology has been commonly adjusted in spite of the use of a large number of mice and avoidable variability in the data. An alternative approach is to collect blood via tail saphenous veins or heart puncture from one mouse for three or four time points (Bateman *et al*., 2001; Kang *et al*., 2009). However, both of these manual serial blood sampling techniques have shortcomings, such as blood loss, labor-intensiveness, the requirement for trained personnel and stress to mice (Jamali and Kunz-Dober, 1999; Peternel *et al*., 2010). In particular, stress influences the normative pharmacokinetics by reducing absorption, delaying gastric emptying time and altering the metabolism (Jamali and Kunz-Dober, 1999; Peternel *et al*., 2010).

Until now, pharmacokinetic studies in mice have been challenging and dependent on individual scientists in spite of their importance. An automated blood sampling (ABS) system was designed to facilitate pharmacokinetic and toxicokinetic studies by automating the collection of multiple blood samples from conscious and freely moving animals without blood loss (Gunaratna *et al*., 2004; He *et al*., 2001; Wang *et al*., 2006). ABS makes it possible to draw an accurate 10 μL of blood at multiple sampling times. Analysis of drugs with low concentrations using only 10 μL of plasma from mice is available using advanced analytical methods such as liquid chromatography tandem mass-spectrometry (LC-MS/MS). However, this system still has difficulty in collecting urine because it vaporizes into air and is smeared into the metabolic cage. Thus, we used the modified mouse urine collector in this study.

As a representative pharmacokinetic study of natural compounds in mice, isoliquiritigenin (isoLQ, 4,2′,4′-trihydroxychalone; Fig. 1) was selected in this study. IsoLQ is a simple chalcone derivative found in licorice, which is one of the most popular herbal medicines with a long history of use around the world. IsoLQ has shown a variety of biological activity including antioxidative (Chin *et al*., 2007; Haraguchi *et al*., 1998), anti-inflammatory (Kim *et al*., 2008), estrogenic (Tamir *et al*., 2001), chemopreventive (Baba *et al*., 2002) and antitumor (Lee *et al*., 2008) effects. In particular, chemopreventive and antitumor effects of isoLQ were reported in *in vivo* and *in vitro* studies: significant anticancer activity in a lung cancer cell line (li *et al*., 2004); suppression of pulmonary metastasis of mouse renal cell carcinoma (Yamazaki *et al*., 2002); induction of apoptosis in human gastric cancer cells (Ma *et al*., 2001); protection via quinone reductase against reactive, toxic and potentially carcinogenic species (Baba *et al*., 2002; Cuendet *et al*., 2006; Ross *et al*., 2000); and prevention of colon and mammary cancer in several rat carcinogenesis models (Baba *et al*., 2002; Wattenberg *et al*., 1994). IsoLQ is metabolized via cytochrome P450 (CYP) isozymes and transformed into butein (via CYP2C19), which also has anticancer activity (Guo *et al*., 2008). Although meaningful pharmacodynamic studies of isoLQ using various cancer xenografted mouse models have already been reported, the pharmacokinetic study of isoLQ in mice has not been reported yet. Thus, it is necessary to determine technical methods for pharmacokinetic studies in mice to investigate the correlation between pharmacokinetic–pharmacodynamic aspects and provide the proper dosage regimen in control as well as cancer xenografted mouse models. Herein, we aimed to establish the ABS technique as a new and general tool by comparing the traditional manual method based on the pharmacokinetics of isoLQ with the LC-MS/MS approach.

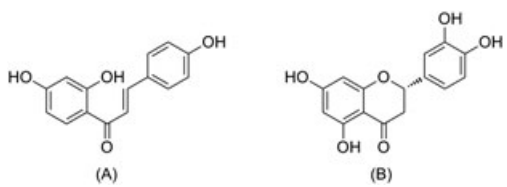

Figure 1. Chemical structures of isoLQ (A) and IS (B).

Experimental

Chemicals

IsoLQ (purity > 98%) was synthesized and purified at the College of Pharmacy, Dongguk University (Goyang, South Korea) according to the previously reported method (Chin *et al*., 2007). Eriodictyol (internal standard, IS) was purchased from Sigma–Aldrich Corp. (St Louis, MO, USA). Polyethylene glycol 400 (PEG 400) was from Showa Chemical Company (Tokyo, Japan). Methanol, acetonitrile, formic acid and 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 were approved by the Institute of Laboratory Animal Resources of Seoul National University, Seoul, South Korea. Male Institute of Cancer Research mice (8 weeks old, weighing 20–30 g) were purchased from Charles River Company Korea (Orient, Seoul, South Korea). Mice were acclimated for one 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 lx. Before conducting this study, mice were allocated to two groups: an ABS group ($n = 5$ for each intravenous and oral study) and a manual group ($n = 5$ for each intravenous and oral study).

Blood sampling instruments and techniques

In ABS group, the automated blood sampling unit (Fig. 2) consisted of a freely moving mice containment device (Raturn™, BASi, West Lafayette, IN, USA), the modified mice urine collector and an automated blood sampler Culex™ (BASi, West Lafayette, IN, USA). The blood samples were collected in a fraction collector HoneyComb™ (BASi, West Lafayette, IN, USA). The CX-2052S and CX-2022S catheters (BASi, West Lafayette, IN, USA) were used for carotid artery and jugular vein cannulation, respectively. In the manual group, 1 mL syringe with a 26 gage needle and a 3 mL syringe with a 32 gage needle are used for intravenous injection of drug via the tail vein and collection of blood by heart puncture, respectively (Kang *et al*., 2009).

In vivo experiments

In ABS group, surgical procedures were conducted under intramuscular injection anesthesia with 125 mg (in 1.5 mL)/kg of tiletamine HCl and zolazepam HCl mixture. The carotid artery (for blood sampling) and the jugular vein (for drug administration) cannulation were carried out using CX-2052S and CX-2022S catheters (BASi, West Lafayette, IN, USA), respectively. Drug administration and blood sampling were started 5 h after surgery.

IsoLQ (dissolved in PEG 400–distilled water, 1:1, v/v) at a dose of 20 mg (in 5 mL)/kg was infused through the jugular vein in the intravenous study after adjusting for catheter dead volume. The ABS system was programmed to collect 10 μL of blood into a micro-vial containing 50 μL of 12.5 units/mL heparinized saline. Blood loss owing to blood sampling was replaced with equal volumes of heparinized saline.

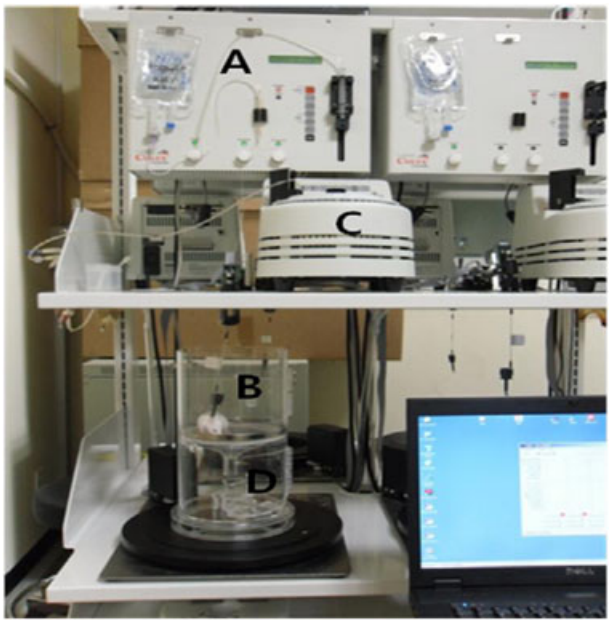

Figure 2. Automated blood sampling unit consisted of an automated blood sampler (A), fraction collector (B), freely moving mouse containment device return (C) and urine collector (D).

Samples were collected at 0, 5, 15, 30, 60, 120, 180, 240, 300 and 360 min after intravenous administration of isoLQ 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 of distilled water and the rinsings were combined with the 24 h urine in a mouse urine collector. At the same time (24 h), each mouse was sacrificed by cervical dislocation, and then the entire gastrointestinal tract (including its contents and feces) was removed and transferred into a beaker that contained 10 mL of methanol (to facilitate the extraction of isoLQ). The gastrointestinal tract was cut into small pieces using scissors. After manual shaking and stirring with a glass rod for 1 min, a 100 μL aliquot of the supernatant were collected from each beaker and stored.

IsoLQ (the same solution as used in the intravenous study) at a dose of 20 mg (in 5 mL)/kg was administered orally using a gastric gavage tube in mice after overnight fasting with free access to water. A blood sample was collected via the carotid artery at 0, 5, 15, 30, 60, 120, 180, 240, 300 and 360 min after the oral administration of isoLQ. Other procedures were similar to those in the intravenous study.

In the manual group, isoLQ (the same solution as used in ABS group) was intravenously administered for 1 min via the tail vein or orally administered using a gastric gavage tube. The blood (0.1 mL) was collected by heart puncture (three points from one mouse) at the same time as the ABS group, and each mouse was sacrificed by cervical dislocation. Other procedures were similar to those in the ABS system.

Analytical instruments and chromatographic conditions

All analytics were performed using an API4000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) controlled by Analyst 1.5.1 software. The mass spectrometer was operated in the multiple reaction monitoring mode with negative ion atmospheric pressure chemical ionization. The multiple reaction monitoring transitions were m/z 254.8 → 118.8 and 287.0 → 150.9 for isoLQ and eriodictyol, respectively. IsoLQ and IS were scanned and dwell times were both 0.15 s. The mass spectra were recorded by electrospray ionization with a negative mode. The turbo ion spray interface was operated at 5500 V and 500°C. The operating conditions, optimized by flow injection of an analyte, were as follows: nebulizing gas flow, 50 psi; curtain gas flow, 20 psi. Quadrupoles Q1 and Q3 were set on unit resolution. Multiple reaction monitoring mode using specific precursor/product ion transition was used for the quantification. The ions were detected by monitoring the transitions of m/z 254.8 → 118.8 and 287.0 → 150.9 for isoLQ (collision energy, –40 eV) and IS (collision energy, –22 eV), respectively.

Solvent A was the distilled water containing 10 mM ammonium acetate and solvent B was methanol. Chromatographic analysis was performed on a XSELECT CSH^{TM} C_{18} (2.1 i.d. × 100 mm; 3.5 μm particle size; Waters, Ireland) main column and C_{18} (4.0 × 3.0 mm; Phenomenex, CA, USA) guard column. An isocratic mobile phase consisting of solvent A and solvent B mixed in the ratio of 15:85 (v/v) was used at a flow rate of 0.25 mL/min. The column oven was maintained at 30°C.

Calibration standards

Stock solutions of isoLQ and IS were prepared in methanol. Serial dilutions of stock solutions of isoLQ (1 mg/mL) were made with methanol at 1000, 100, 10, 1 and 0.5 μg/mL. A 10 μL of each isoLQ stock solution was spiked into 1 mL of mouse whole plasma for the calibration standards in manual group. In the ABS group, 10 mL mouse whole blood and 50 mL of 12.5 units/mL heparinized-saline were mixed and the supernatant was collected after centrifugation. A 10 μL of each isoLQ stock solution was spiked into 1 mL of mouse plasma with heparinized-saline. The concentrations of calibration standards were 10, 1, 0.1, 0.01 and 0.005 μg/mL in both groups of mice. After mixing each concentration of calibration standard in both groups, 1 mL calibration standards in both groups were divided into 50 μL in each tube. A 200 μL acetonitrile containing 50 ng/mL IS was added to 50 μL of a sample. After vortex-mixing for 1 min and centrifugation (15,000***g*** for 10 min), 50 μL of the supernatant was directly injected onto the column. In urine and gastrointestinal tract samples, the calibration standards were prepared in the same way as the plasma calibration standards.

In both groups, the lower limit of detection (LLOD) was defined as a signal-to-noise ratio of >10, whereas the LLOD was defined as a coefficient of variation (CV, %) of <15%. Four sets of validation samples at concentrations of 10, 1, 0.1, 0.01 and 0.005 μg/mL were used to construct calibration curves. Similarly, inter- and intraday validations were performed to validate the precision and accuracy of the assay. For inter- and intra-day validations, four sets of control samples at different concentrations of 10, 1, 0.1, 0.01 and 0.005 μg/mL were evaluated. The best linear fit and least squares residuals of the calibration curve were achieved using a 1/concentration^2 weighing factor with a mean linear regression equation. The calibration curve was $y = ax + b$, where y is the peak ratio of isoLQ to IS and x is the concentration of isoLQ.

Sample preparation

Sample preparations involved simple protein precipitation with acetonitrile. The same as the preparation of calibration standards, a 200 μL acetonitrile containing 50 ng/mL IS was added to 50 μL of a sample. After vortex-mixing for 1 min and centrifugation (15,000***g*** for 10 min), a 50 μL of the supernatant was directly injected onto an XSELECT CSH^{TM} C_{18} (2.1 × 100 mm, 3.5 μm particle size; Waters, Ireland) column.

Pharmacokinetic analysis

The total area under the plasma concentration–time curve from time zero to infinity (AUC) was calculated using the trapezoidal rule-extrapolation method (Chiou, 1978). The area from the last datum point to time infinity was estimated by dividing the last measured plasma concentration by the terminal-phase rate constant.

Standard methods (Gibaldi and Perrier, 1982) were used to calculated the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin 2.1; Pharmasight Corp., Mountain View, CA, USA): the time-averaged total body, renal and non-renal clearances (CL, CL_R and CL_{NR}, respectively), terminal half-life, mean residence time and apparent volume of distribution at steady state (Kim *et al*., 1993). The extent of absolute oral

bioavailability (F) was calculated by 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

Student's t-test was used to compare pharmacokinetic parameters determined between two methods. Statistical significance was accepted for a p-value of < 0.05. Results are expressed as means $\pm$ standard deviations.

Results

Selection of internal standard

We investigated several compounds to find a suitable IS, and chose a compound capable of being resolved to the endogenous peak and determined simultaneously with isoLQ. In order to develop an analytical method with the desired LLOD (5 ng/mL), it is necessary to use MS/MS detection because the MS/MS

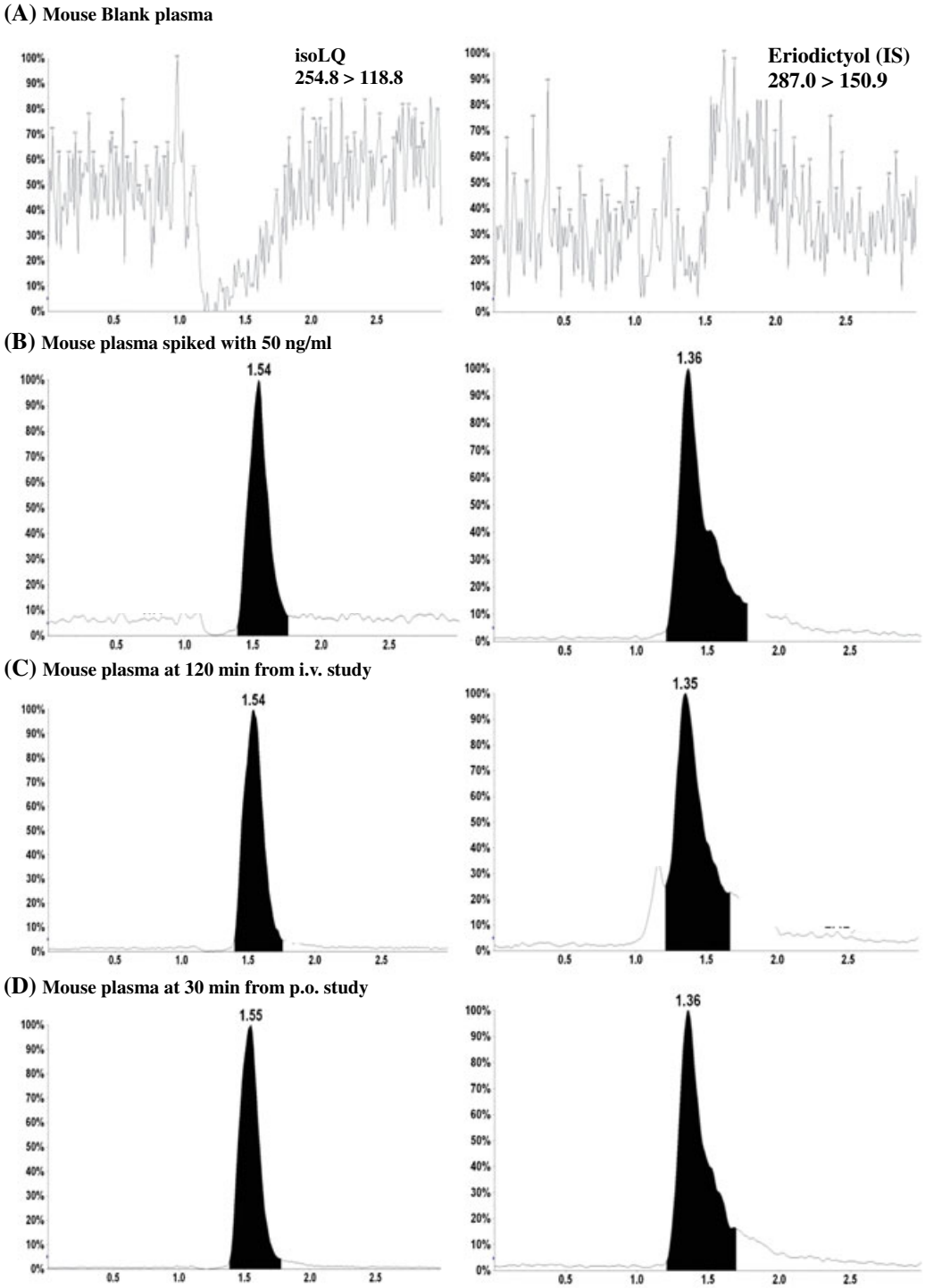

Figure 3. Mass chromatograms after deprotenization of plasma sample with acetonitrile containing IS for the mouse blank plasma (A), mouse plasma spiked with 50 ng/mL isoLQ (B) and mouse plasma samples at 120 min in i.v. study (C) and 30 min in p.o. study (D) in manual group.

analytical method provides the very low limit of detection (LOD) required for trace mixture analysis (Jemal *et al*., 2010). The full-scan negative mass spectra of isoLQ and IS ($[M-H]^-$) at 118.8 and 150.9, respectively, in the Q1 spectrum were used as precursor ions to lose product ion spectra. No interferences were observed for isoLQ and IS when measuring the *m/z* 254.8 → 118.8 and 254.8 → 150.9 transitions, respectively.

Validation results

Chromatographic conditions, especially the composition of the mobile phase, were optimized to achieve a good resolution, symmetrical peak shapes for isoLQ and IS, acceptable retention factors ($k' \geq 2$) and a short run time. Isocratic mobile phase consisting of solvent A (purified water containing 10 mM ammonium

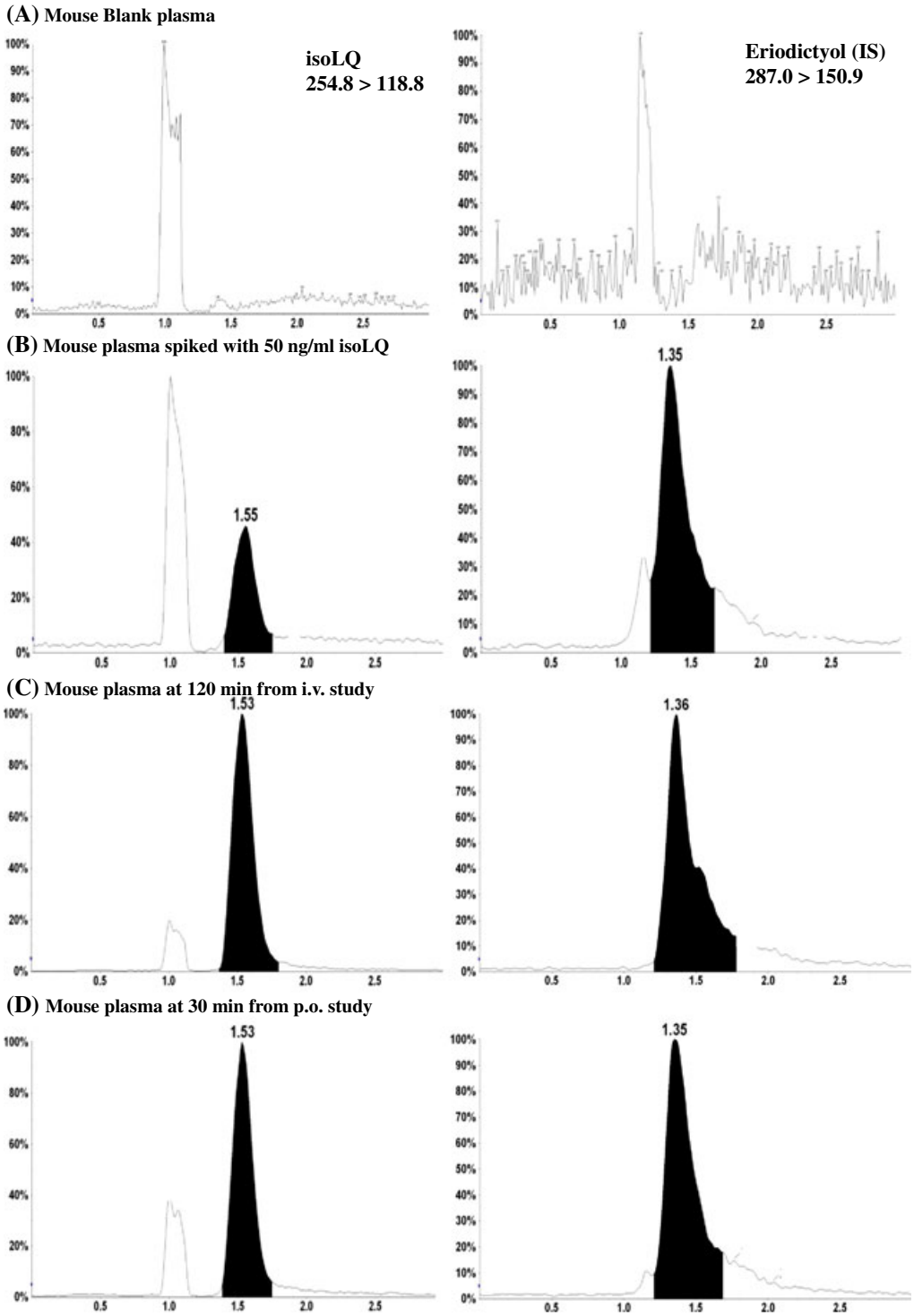

Figure 4. Mass chromatogram after deprotenization of plasma sample with acetonitrile containing IS for the mouse blank plasma (A), mouse plasma spiked with 50 ng/mL isoLQ (B) and mouse plasma samples at 120 min in i.v. study (C) and 30 min in p.o. study (D) in ABS group.

acetate) and solvent B (methanol) mixed at the ratio 15:85 (v/v, A/B) was found to be suitable. A flow rate of 0.25 mL/min was required to obtain the retention times of 1.54 and 1.35 min for isoLQ and IS, respectively. Ammonium acetate was found to be necessary in order to lower pH and thereby to produce a symmetrical peak shape at a satisfactory retention factor. The percentage of ammonium acetate was also optimized to achieve a symmetrical peak shape, good ionization and fragmentation.

The calibration curve constructed for isoLQ in plasma for ABS group was linear over the concentration range 5–10,000 ng/mL. The calibration curve was $y = 0.00710x - 0.00400$, and the correlation coefficient (r^2) was 0.999. The inter- and intra-day precisions were expressed as CV (%) and were <15% (maximum 12.9% and minimum 0.0731% for an LLOD sample), and the accuracy was between 97.3 and 103%, which complies with the FDA regulations (US Deparment of Health and Human services, Food and Drug Administration and Center for Drug Evaluation and Research, 2001). The calibration curve constructed for isoLQ in plasma for the manual group was also linear over the concentration range 5–10,000 ng/mL, giving a mean linear regression equation for the calibration curve of $y = 0.0101x + 0.00570$, and the r^2 was 0.998. Inter- and intra-day precisions were measured, and ranged from 1.20 to 9.53% for LLOD samples and accuracy from 97.2 to 103%.

Mass chromatograms of mouse blank plasma, mouse plasma spiked with 10 ng/mL isoLQ and mouse plasma collected from i.v. and p.o. studies after the deprotenization procedure with acetonitrile containing IS for the manual and ABS groups are shown in Figs 3 and 4, respectively. These results demonstrated that the deproteinization method is simple and suitable for the quantification of isoLQ in mouse plasma; hence it was successfully implemented for the analysis of plasma samples. Validation data are summarized in Table 1.

Table 1. Validation of the LC-MS/MS method for measuring isoLQ in mouse plasma obtained using the ABS and manual methods

Parameter	ABS ($n = 4$)	Manual ($n = 4$)
Lower limit of detection (ng/mL)	5	5
Calibration range (ng/mL)	5–10,000	5–10,000
Calibration equation	$y = 0.00710x$ – 0.00400	$y = 0.0101x +$ 0.00570
Calibration coefficient (r^2)	0.999	0.998
Inter-day precision (CV, %, $n = 4$)		
5 ng/mL	2.16	1.45
10 ng/mL	3.29	2.79
100 ng/mL	1.75	1.14
1000 ng/mL	1.45	0.944
10,000 ng/mL	2.43	2.42
Inter-day accuracy (%, $n = 4$)		
5 ng/mL	110.76	110.68
10 ng/mL	105.41	105.33
100 ng/mL	100.36	103.71
1000 ng/mL	100.16	100.26
10,000 ng/mL	100.41	100.06
Intra-day precision (CV, %, $n = 4$)		
5 ng/mL	0.645	1.58
10 ng/mL	1.14	2.92
100 ng/mL	1.43	1.15
1000 ng/mL	0.945	0.943
10,000 ng/mL	2.43	2.46
Intra-day accuracy (%, $n = 4$)		
5 ng/mL	94.51	93.13
10 ng/mL	97.48	96.64
100 ng/mL	99.91	99.75
1000 ng/mL	100.01	99.89
10,000 ng/mL	100.01	99.90

Intravenous studies

After the intravenous administration of isoLQ at a dose of 20 mg/kg to two groups of mice, its mean arterial plasma concentration–time profiles are shown in Fig. 5. The relevant pharmacokinetic parameters are listed in Table 2. In the manual group, the AUC was significantly greater (54.7% increase) than in the ABS group, while *CL*, CL_R and CL_{NR} were significantly slower (33.3, 28.1 and 33.8% decrease).

Oral studies

After the oral administration of isoLQ at a dose of 20 mg/kg to two groups of mice, its mean arterial plasma concentration–time profiles are shown in Fig. 6. The relevant pharmacokinetic parameters are listed in Table 3. After oral administration of isoLQ, isoLQ was detected in plasma from the first blood sampling time point (5 min), suggesting that the absorption of isoLQ in the gastrointestinal tract is rapid. In manual group, the AUC (31.7% increase) and the dose recovered from the entire gastrointestinal tract (including its contents and feces) at 24 h ($GI_{24\ h}$; 91.6% increase) were significantly greater than ABS group, whereas T_{max} was significantly slower (1100% increase).

Discussion

The full-scan negative mass spectra of isoLQ and IS exhibited deprotonated mass ions in the Q1 spectrum. These ions were used as precursor ions to obtain product ion spectra, and were well resolved in analysis of isoLQ in animal plasma. In both analytical procedures, the inter- and intra-day precisions were <15%, expressed as CV (%), and the accuracy was between 80 and 120% (Table 1), which complies with the FDA regulations (US Deparment of Health and Human services, Food and Drug Administration and Center for Drug Evaluation and Research, 2001).

The contribution of gastrointestinal excretion of unchanged isoLQ to its CL_{NR} was almost negligible; the percentages of the dose recovered from the entire gastrointestinal tract (including its contents and feces) at 24 h ($GI_{24\ h}$) were only 1.30 and 2.41% of the intravenous dose for ABS and manual groups, respectively (Table 2). However, the low values of $GI_{24\ h}$, that is, 1.30 and 2.41%, were probably not due to chemical and enzymatic degradation of isoLQ in gastric fluids; isoLQ was stable up to 24 h incubation in various buffer solutions having a pH ranging from 4 to 10 and in GI samples from mice (at least 90.7% of spiked isoLQ was remaining in all samples; our unpublished data). Thus, the CL_{NR} of isoLQ could represent its metabolic clearances.

The *CL* of isoLQ (230–345 mL/min/kg; Table 2) were somewhat and considerably faster than the cardiac output (220 mL/min/kg)

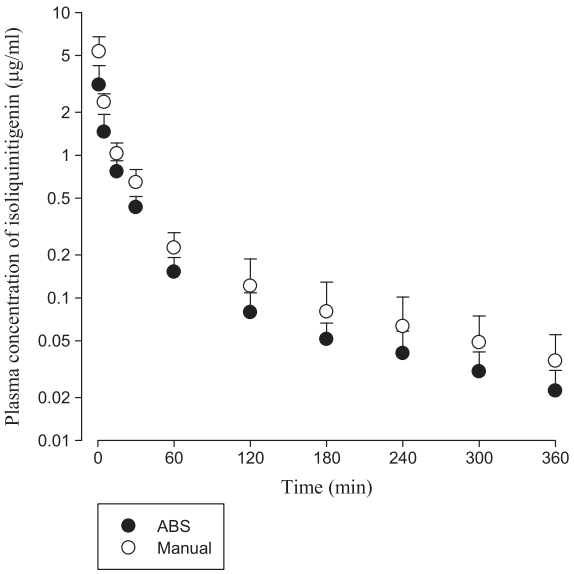

Figure 5. Mean plasma concentration–time profiles of isoLQ after i.v. administration of isoLQ obtained from ABS (●) and manual (○) groups. Bars represent standard deviations.

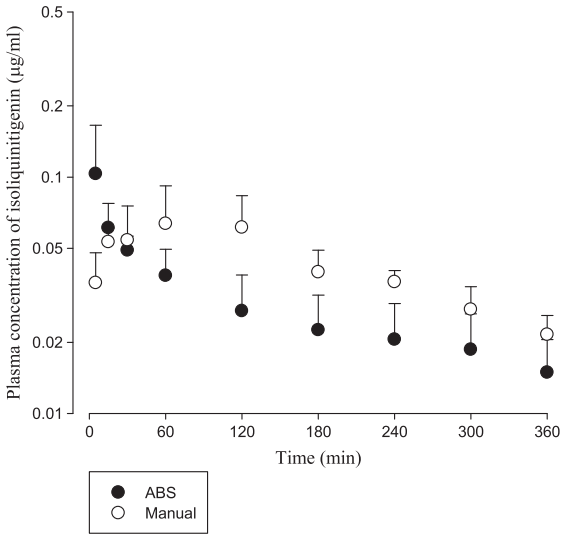

Figure 6. Mean plasma concentration–time profiles of isoLQ after p.o. administration of isoLQ obtained from ABS (●) and manual (○) groups. Bars represent standard deviations.

Table 2. Mean (± SD) pharmacokinetic parameters of isoLQ after intravenous administration of isoLQ at a dose of 20 mg/kg to mice by ABS and manual methods

Parameter	ABS ($n = 5$)	Manual ($n = 5$)
Body weight (g)	31.0 ± 2.24	29.0 ± 4.18
AUC (μg min/mL)	59.2 ± 9.48	91.6 ± 25.3[a]
Terminal half-life (min)	145 ± 14.8	171 ± 30.5
MRT (min)	101 ± 25.3	109 ± 15.1
CL (mL/min/kg)	345 ± 59.0	230 ± 52.8[a]
CL_R (mL/min/kg)	11.5 ± 3.54	8.27 ± 3.82[a]
CL_{NR} (mL/min/kg)	334 ± 57.1	221 ± 50.3[a]
V_{ss} (mL/kg)	34400 ± 7220	34600 ± 3640
$Ae_{0-24\ h}$ (%)	3.35 ± 0.818	3.54 ± 1.14
$GI_{24\ h}$ (%)	1.30 ± 1.16	2.41 ± 1.71

[a]Significantly different ($p < 0.05$) from ABS group.
MRT, Mean residence time; CL, C_R and CL_{NR}, the time-averaged total body, renal and non-renal clearances, respectively; V_{ss}, apparent volume of distribution at steady state; $GI_{24\ h}$, dose recovered from the entire gastrointestinal tract (including its contents and feces) at 24 h.

Table 3. Mean (± SD) pharmacokinetic parameters of isoLQ after oral administration of isoLQ at a dose of 20 mg/kg to mice by ABS and manual methods

Parameter	ABS ($n = 5$)	Manual ($n = 5$)
Body weight (g)	28.3 ± 2.89	27.0 ± 2.74
AUC (μg min/mL)	16.4 ± 4.33	21.6 ± 2.78[a]
Terminal half-life (min)	234 ± 10.2	181 ± 61.7
CL_R (mL/min/kg)	19.2 ± 9.35	18.2 ± 7.90
C_{max} (μg/mL)	0.103 ± 0.0625	0.0656 ± 0.0313
T_{max} (min)	5 (5–5)	60 (5–120)[b]
$Ae_{0-24\ h}$ (% of dose)	0.923 ± 0.329	1.26 ± 0.376
$GI_{24\ h}$ (% of dose)	3.44 ± 4.48	6.59 ± 2.42[a]
F_{abs} (%)	96.8	93.8
F (%)	27.7	23.6

[a]Significantly different ($p < 0.05$) from ABS group.
[b]Significantly different ($p < 0.01$) from ABS group.
F, Oral bioavailability. Fabs: unabsorbed fraction.

and hepatic blood flow rate (49.5 mL/min/kg), respectively, based on the plasma data (using hematocrit of 0.45 in mouse; Davis and Morris, 1999). The above data indicate that isoLQ was metabolized in the extra-hepatic organs in mouse. This was proved by *in vitro* metabolism studies using tissue homogenates: 45.3, 21.8, 14.3, 5.33 and 28.9% of spiked isoLQ disappeared [mainly metabolized after 30 min incubation of 1 μg/mL isoLQ with 9000***g*** supernatant (S9) fraction of liver, small intestine, kidney, lung and heart, respectively (our preliminary data)]. It has been also reported that heart is one of the organs involved in the presystemic metabolism via cytochrome P450 and UDP-glucuronyltransferase (Guo *et al.*, 2008) and these enzymes are expressed in heart (Heide *et al.*, 2004; Wu et *al.*, 1997).

The values of CL_R of isoLQ were estimated based on free (unbound to plasma proteins) fractions (14.0 and 16.3% for ABS and manual groups, respectively; our unpublished data) in plasma ($CL_{R,\ fu}$); the $CL_{R,\ fu}$ values thus estimated were 82.1 and 50.7 mL/min/kg for ABS and manual groups, respectively. The values were considerably faster than glomerular filtration rates, 14.0 mL/min/kg, in mouse (based on the creatinine clearance; Davis and Morris, 1999). This suggests that isoLQ is excreted into urine predominantly via active renal tubular secretion in mouse.

The F-values of isoLQ in mice were low, only 23.6–27.7% (Table 3). To determine whether the poor gastrointestinal

absorption of isoLQ caused the low *F* or not, the 'true' fraction of oral dose of isoLQ unabsorbed (F_{unabs}) was calculated based on the reported equation assuming linear pharmacokinetics (Lee and Chiou, 1983):

$$0.0344 = F_{unabs} + (0.277 \times 0.0130) \text{ for the ABS group}$$
$$0.0659 = F_{unabs} + (0.236 \times 0.0241) \text{ for the manual group}$$

in which 0.0344 (0.0659), 0.0130 (0.0241) and 0.277 (0.236) are the fraction of the dose remaining in the whole gastrointestinal tract at 24 h (including its contents and feces) of the oral and intravenous dose, respectively, and *F*, respectively, for ABS (manual) groups. The F_{unabs} values thus estimated were 0.0308 and 0.0602 for the ABS and manual groups, respectively. Thus, the absorbed fractions of isoLQ were 0.969 and 0.940 in the ABS and manual groups, respectively. The above data indicate that the low *F* of isoLQ was not due to the poor gastrointestinal absorption of isoLQ. The low *F* of isoLQ could be due to considerable gastrointestinal and/or hepatic first-pass effect.

The AUC and each clearance values of isoLQ were significantly different between the ABS and manual groups (Tables 2 and 3). This could be mainly due to the difference in blood sampling site (heart puncture for manual group and carotid artery for ABS group) between the two groups (Chiou, 1989a, 1989b). It has been reported that the driving force for distribution and elimination of drugs is the concentration in arterial blood (Chiou, 1989a). Thus, the pharmacokinetic data using arterial blood (ABS technique) is more reliable. However, a contribution of differences in immobilization stress to different AUCs and each clearance of isoLQ between the two groups could not be totally ruled out (He *et al*., 2001; Gunaratna *et al*., 2004; Zhu *et al*., 2005).

Multiple blood sample collections from a single animal with minimal blood loss (Mitruka and Rawnsley, 1987) and minimal stress is important during pharmacokinetic studies (Heta *et al*., 1986). The common problems associated with traditional blood sampling are catheter occlusion by blood clots (thus the continuous flushing of catheters with heparinized saline is required to prevent clots, which also increases fluid volume in plasma and changes in blood pressure and immobilization stress). When the ABS method is employed, mice are able to move freely and stress can be minimized. Although the surgical procedure induces emotional and immobilization stress even using the ABS method, uniform tending of catheters, their regular diameters cannulated into the veins or arteries and their patencies could help prevent stress (Wang *et al*., 2006). Also, the ABS method collected the blood samples from the same site differently from that there is no guarantee of blood sampling site.

In conclusion, the *F* of isoLQ was low, 27.7 and 23.6%, and this could be due to a considerable gastrointestinal and/or hepatic first-pass effect of isoLQ in mouse. The differences in AUC and each clearance of isoLQ between ABS and manual groups could be mainly due to the difference in blood sampling site and less to stress. The ABS method has many advantages over the manual method. Thus, the ABS method is a useful drug development tool that can reduce the blood volume for blood collection and the time required for preclinical *in vivo* studies. In addition, the ABS method provides reliable and accurate pharmacokinetic parameters from small numbers of mice by minimizing the shortcomings of the manual technique (e.g. inter-animal and physiological variations).

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Conflict of interest

None declared.

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