

**PHARMACOKINETICS AND METABOLISM OF LIGUSTILIDE, A MAJOR  
BIOACTIVE COMPONENT IN RHIZOMA CHUANXIONG, IN THE RAT**

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**Running title:**           **Pharmacokinetics of ligustilide in rat**

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- a) **Abbreviations used are:** GSH, glutathione; SGF, simulated gastric fluid; SIF simulated intestinal fluid; TCM, traditional Chinese medicine; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; UV, ultraviolet; MS, mass spectrometry; DAD, diode array detector; LOQ, limit of quantification; RSD, relative standard deviation

**ABSTRACT:**

Ligustilide is the most abundant bioactive ingredient in *Rhizoma Chuanxiong*, a Chinese medicinal herb commonly used for the treatment of cardiovascular ailments. The present study reported, for the first time, the pharmacokinetics of ligustilide, administered in its pure form and in an herbal extract, in rats. After intravenous administration of pure ligustilide, it was distributed extensively ( $V_d$ :  $3.76 \pm 1.23$  L/kg) and eliminated rapidly ( $t_{1/2}$ :  $0.31 \pm 0.12$  hr). The intravenous clearance of ligustilide, after *Chuanxiong* extract administration, was significantly higher than that dosed in its pure form (CL:  $20.35 \pm 3.05$  vs  $9.14 \pm 1.27$  L/hr/kg,  $p < 0.01$ ; AUC:  $0.79 \pm 0.10$  vs  $1.81 \pm 0.24$  mg·hr/L,  $p < 0.01$ ), suggesting significant interaction between ligustilide and components present in the extract. Dose-dependent pharmacokinetics was observed after intraperitoneal administration, and a significantly higher dose-normalized AUC ( $1.77 \pm 0.23$  mg·hr/L) at 52 mg/kg was obtained than that at 26 mg/kg ( $0.93 \pm 0.07$  mg·hr/L,  $p < 0.05$ ). Oral bioavailability of ligustilide was low (2.6%), which was partly due to extensive first-pass metabolism in the liver. Seven metabolites of ligustilide were identified, and three of them were unequivocally characterized as butylidenephthalide, senkyunolide I and senkyunolide H. These three compounds also occurred naturally in the herb and were reported to be bioactive.

Rhizoma Chuanxiong (*Ligusticum chuanxiong* Hort. (Umbelliferae)), known as Chuanxiong in Chinese, a commonly used Chinese medicinal (CM) herb, has been used for treating cardiovascular diseases in China for centuries (China Pharmacopoeia, 2005). In addition to raw herb preparations used in conventional CM practice, various Chuanxiong-based proprietary products containing Chuanxiong crude extract, especially Chuanxiong essential oil extract, are also used clinically. For instance, Quick-acting Heart Saving Pill (Chinese name: Suxiao Jiuxin Wan), a product manufactured for sublingual delivery, contains Chuanxiong essential oil extract as the primary ingredient. In recent years, this product is one of the top five best-selling CM-based cardiovascular products in China (Li, 2005; Sun et al., 2002).

In the past decades, the phytochemistry of Chuanxiong has been studied extensively. Nineteen compounds from *L. chuanxiong* have been reported to be pharmacologically active (Naito et al., 1995; Matsumoto et al., 1998; Ko et al., 1997, 1998, 2002; Chong and Feng, 1999; Shih et al., 2002). In particular, phthalides are present in large quantities in Chuanxiong essential oil; these compounds have been shown to have *in vitro* and *in vivo* pharmacological activities (Chan et al., 2006, 2007; Du et al., 2006; Cao et al., 2006; Lu et al., 2006; Matsumoto et al., 1998; Naito et al., 1995; Ko et al., 1998, 2002; Chong and Feng, 1999). Ligustilide (Z-3-butylidene-4,5-dihydroisodenzofuranone, Fig. 1), a phthalide derivative, is the most abundant constituent in the herb (Yan et al., 2005; Li et al., 2002; Li et al., 2006). Vasodilatation, anti-platelet aggregation, anti-thrombotic, serotonergic activity and anti-proliferative properties of ligustilide have been well documented (Cao et al., 2006; Chan et al., 2007; Du et al., 2006; Naito et al., 1995; Lu et al., 2006; Deng et al., 2006). Hence, ligustilide is considered to be a key active ingredient in Chuanxiong.

Like many medicinal herbs, most Chuanxiong products are taken orally in CM practice; however, to date, pharmacokinetics of Chuanxiong ingredients are largely unknown and the justification for its mode of administration is unavailable. As part of a systematic investigation of this CM herb, our research team has investigated the pharmacokinetics of major Chuanxiong ingredients. Our recent study in rats showed that senkyunolide A, another bioactive Chuanxiong phthalide, was unstable in the gut and undergoes extensive first-pass metabolism in the liver, leading to a very low oral bioavailability (Yan et al., 2007). Based on these findings, the effectiveness of oral administration of Chuanxiong is limited. Metabolism of ligustilide has not been reported and data on pharmacokinetics of ligustilide are scanty. Shi et al (2006) studied pharmacokinetics of ligustilide after oral dose. The study was not designed to estimate absolute bioavailability; therefore, the potential problem with absorption was not addressed.

The present studies investigated pharmacokinetics of ligustilide in its pure form and in a Chuanxiong extract, and also evaluated the effects of administration routes on pharmacokinetics of ligustilide. Furthermore, *in vitro* studies were conducted to study ligustilide metabolism in greater details.

## Materials and Methods

**Chemicals** Methanol and acetonitrile were HPLC grade from Fisher Scientific (Leicestershire, Irish, UK). *z*-Ligustilide (ligustilide, 99% pure, structure shown in Fig. 1) in DMSO (10 mg/mL) was purchased from ChromaDex (Irvine, CA, USA), while *n*-butylidenephthalide (butylidenephthalide, 96% pure) and all other chemicals were from Sigma (St. Louis, Mo, USA). Chuanxiong crude oil extract (Chuanxiong extract) prepared

using CO<sub>2</sub> supercritical extraction was obtained from Masson Pharmaceutical Ltd. (Guangzhou, China). Senkyunolide I (99% pure) and senkyunolide H (94% pure) were isolated from Chuanxiong extract in our laboratory (Li et al., 2003). Concentration of the only commercially available ligustilide was too low for *in vivo* studies. This preparation was, therefore, utilized in the *in vitro* metabolic and stability studies only. For the *in vivo* studies, ligustilide was isolated from Chuanxiong extract in our laboratory using a previously developed method (Li et al., 2003). Due to the instability of ligustilide in its pure form as reported previously (Cui et al., 2006; Lin et al., 1998), the chromatographically ‘purified’ ligustilide also contained 5% of butylidenephthalide. Identities of all isolated compounds were confirmed by matching their retention times, UV and MS spectra with those of the authentic standards using our previously developed HPLC-UV-MS method (Li et al., 2003) and their purities were determined using the HPLC-UV method developed by our group (Yan et al., 2005). Furthermore, contents of ligustilide, butylidenephthalide, and other phthalides in Chuanxiong extract (Table 1) were also determined using the same manner. The ratio of ligustilide and butylidenephthalide in Chuanxiong extract was found to be 18:1, similar to that found in the ‘purified’ ligustilide.

***In vivo* Protocols** Male Sprague-Dawley (SD) rats (200-230 g) were supplied by the Laboratory Animal Service Center, The Chinese University of Hong Kong. Animals were housed under standard conditions of temperature, humidity and light. Food and water were provided *ad libitum*. A well-established *in vivo* pharmacokinetic model in the rat (Su et al., 2006; Yan et al., 2007) was adopted in the present study. The day before administration, a jugular vein cannula was implanted for blood sampling under light anesthesia with diethyl ether. Rats were divided randomly into five groups (5 animals in each group). For animals

receiving intravenous dosing, a femoral vein cannula was also implanted. After cannulation, rats were allowed to recover and fasted overnight with free access to water. For intravenous (iv) administration, the 'purified' ligustilide sample (ligustilide dose: 15.6 mg/kg) or Chuanxiong extract (100 mg/kg, ligustilide dose: 14.9 mg/kg) formulated in Pharmatek™ formulation-6 (Pharmatek, San Diego, CA, USA) was injected into the left femoral vein through the catheter. A single dose of ligustilide formulated in normal saline containing 3% Tween-80 was given orally (100, 360 or 500 mg/kg) or intraperitoneally (ip) (26 or 52 mg/kg) to individual groups of rats. Blood (~0.3 mL/sample) was collected into heparinized Eppendorf tubes at appropriate time intervals over a 48-hr period as illustrated in Fig. 2. After each collection, 0.3 mL of saline containing 40 IU heparin/mL was injected to compensate for blood loss. Blood samples were centrifuged at  $3000 \times g$  for 10 min and plasma samples were harvested. Each plasma sample (160  $\mu$ L) was mixed with 5  $\mu$ L of internal standard ( $\alpha$ -naphthaflavone, 40  $\mu$ g/mL) and 165  $\mu$ L of acetonitrile, vortexed and centrifuged at  $16000 \times g$  for 5 min. The supernatant was filtered with a 0.45  $\mu$ m syringe filter, and 100  $\mu$ L of the filtrate was subjected to HPLC analysis.

***In vitro* Metabolic Study** Pooled (5 untreated male SD rats) liver and small intestine homogenates (S9) and microsomes were prepared using standard methods (Lin et al., 2000; 2007; Williams et al., 1989). Total contents of protein and cytochrome P450 were measured using the Lowry (Lowry et al., 1951) and the Omura method (Omura and Sato, 1964), respectively. The reaction components contained one of the subcellular preparations (2 mg protein/mL) described above, ligustilide (5 to 800  $\mu$ M), NADPH-regenerating system (1 mM NADP<sup>+</sup>, 1 mM NAD<sup>+</sup>, 10 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 4 mM MgCl<sub>2</sub>), 50 mM Tris buffer containing 150 mM of KCl

(pH 7.4) in a total volume of 1 mL. There were three types of control samples: one contained no NADPH-regenerating system; one had neither substrate nor NADPH-regenerating system and one had the protein of the subcellular fraction denatured by heat. Reactions were initiated by the addition of NADPH-regenerating system, or substrate in case of the control containing no NADPH-regenerating system. The mixture was incubated for 1 hr at 37 °C with gentle shaking. The reaction was terminated by the addition of an equal volume of acetonitrile containing the internal standard; the mixture was kept on ice for 5 min. The resultant sample was centrifuged, filtered, and 100  $\mu$ L of filtrate was subjected to HPLC analysis. All controls and reactions were processed in parallel. Each sample was processed in triplicate.

**Stability of Ligustilide in Simulated Gastric and Intestinal Fluids** One volume of ligustilide (1 mg/mL) in 3% Tween-80 saline solution was spiked into three volumes of simulated gastric fluid (SGF) containing no pepsin [0.05M sodium chloride adjusted to pH 1.5 using HCl] (USP, 2006) or simulated intestinal fluid (SIF) containing no pancreatin [0.05M sodium dihydrogen phosphate buffer adjusted to pH 6.8 using NaOH] (USP, 2006). The mixtures were incubated for 1 hr at 37 °C. Aliquots (100  $\mu$ L) of incubates were collected at 0, 5, 15, 30 and 60 min. Each aliquot was immediately extracted with ethyl acetate (200  $\mu$ L), followed by centrifugation at 500  $\times$  g for 5 min. After removing the organic layer, the sample was extracted again with the same procedure. The organic layers were combined and an aliquot (150  $\mu$ L) was mixed with 150  $\mu$ L of methanol containing 1.75  $\mu$ g of  $\alpha$ -naphthaflavone. The resultant solution was filtered and an aliquot (100  $\mu$ L) of the filtrate was subjected to HPLC analysis. Each test was conducted in triplicate. Degradation of ligustilide in the simulated gastric and intestinal fluids was determined by



comparing peak area ratios (ligustilide/ $\alpha$ -naphthaflavone) of the samples with that at time zero.

**Identification of Ligustilide and Its Metabolites** Ligustilide and its metabolites senkyunolide I (L1), senkyunolide H (L2) and butylidenephthalide (L13) in biological samples were unambiguously identified by comparing their retention time, MS and UV spectra with those of authentic compounds. Identities of other metabolites were tentatively elucidated using an on-line HPLC-UV-MS method as described below.

The chromatographic condition was the same as described in the section of Quantitative Analysis. The on-line HPLC-UV-MS analysis was performed on a Perkin-Elmer series 200 liquid chromatograph connected to a Perkin-Elmer Sciex API-2000 triple-quadrupole mass spectrometer (PerkinElmerSciex Instruments, Boston, MA, USA) equipped with electrospray ionization (ESI) interface. Positive ESI-MS was conducted under the following conditions: nebulizing gas 40 psi, auxiliary gas 60 psi, curtain gas 30 psi, turboionspray temperature 400 °C, declustering potential 61 V, focusing potential 380 V, and ionization potential 5500 V. Full scan mass spectrum was obtained over a range of  $m/z$  150-700. Mass spectrum was recorded with a PE Sciex MassChrom data system (version 1.1.1).

**Quantitative Analysis** *HPLC-UV method* The HPLC system consisted of an Agilent series 1100 HPLC-DAD (Agilent Technologies, Wilmington, USA) and a Waters Symmetry C<sub>18</sub> column (5  $\mu$ m, 150  $\times$  4.6 mm) coupled with a guard column (Waters Spherisorb S5 ODS2, 10  $\times$  4.6 mm). The samples were eluted using a mobile phase containing A (0.25% aqueous acetic acid, v/v) and B (methanol). A previously reported gradient system was adopted for the analysis of both *in vivo* and *in vitro* samples (Yan et

al., 2005). Ligustilide was monitored at wavelength of 284 nm.

*Calibration curves of ligustilide* A methanol stock solution of ligustilide was diluted to the desired concentrations with methanol. Aliquot (5  $\mu$ L) of each diluted solution was spiked into pooled plasma (3 untreated male SD rats) to give concentrations ranging from 0.2 to 25 mg/L (0.2, 0.8, 2, 4, 8, 16 and 25 mg/L) for the *in vivo* study, or into Tris buffer to give a concentration ranging from 1.9 to 95 mg/L for the *in vitro* study. The resultant samples were mixed thoroughly, then treated and analyzed in the same manner as described in the section for the *In Vivo* Protocols. Samples of each concentration were analyzed in triplicate. Calibration curves were derived by plotting the peak area ratios of ligustilide to the internal standard as a function of concentration of ligustilide.

*Method validation* Three concentrations of ligustilide at high, medium and low levels of the corresponding calibration curves were chosen to determine the intra- and inter-day variability. Samples at each concentration were analyzed in triplicates to examine intra-day variability. The inter-day variability was determined on three separate days over a period of one week. Peak area ratio for each concentration was measured, and concentration of ligustilide was calculated from the corresponding calibration curve. Relative standard deviation (RSD) and percentage difference between amounts spiked and determined (recovery) were taken as measures of precision and accuracy, respectively.

Aliquots of methanol stock solution were also spiked into blank plasma or incubation buffer to determine limits of quantification (LOQ) of ligustilide. The resultant samples were treated and analyzed using the procedures described in this section. LOQ was determined when the concentration of a sample has a signal-to-noise ratio at or higher than 5.

**Analysis of Pharmacokinetic Parameters** Pharmacokinetic parameters were calculated from the corresponding plasma concentration-time curves using non-compartmental analysis (WinNonlin 4.0, Pharsight Corp., CA, USA). The area under the plasma concentration-time curve from time zero to time infinity ( $AUC_{0 \rightarrow \infty}$ ) was calculated using the linear/log trapezoidal rule-extrapolation method, in which the linear trapezoidal rule was employed for the calculation of the area during the ascending phase with the logarithmic trapezoidal rule for the declining phase.  $C_{max}$  and  $T_{max}$  were determined from the concentration-time profile. Other pharmacokinetic parameters, including apparent plasma clearance ( $CL/F$ ), apparent volume of distribution based on the terminal phase ( $V_d/F$ ), mean residence time (MRT) and absolute bioavailability (F) were calculated using the following equations:

$$CL/F = \text{Dose}/AUC_{0 \rightarrow \infty}$$

$$V_d/F = \text{Dose}/(\lambda_z \times AUC_{0 \rightarrow \infty})$$

$$MRT = AUMC_{0 \rightarrow \infty}/AUC_{0 \rightarrow \infty}$$

$$F = (AUC_{\text{oral/ip}} \times \text{Dose}_{\text{iv}}) / (AUC_{\text{iv}} \times \text{Dose}_{\text{oral/ip}})$$

Where  $\lambda_z$  is the terminal rate constant;  $AUMC_{0 \rightarrow \infty}$  is the area under the first moment curve from time 0 to infinity;  $\text{Dose}_{\text{iv}}$  is the intravenous dose of ligustilide;  $\text{Dose}_{\text{oral/ip}}$  is the oral or intraperitoneal dose of ligustilide.  $AUC_{\text{iv}}$  is the area under the plasma concentration-time curve of ligustilide after intravenous administration;  $AUC_{\text{oral/ip}}$  is the corresponding area under the plasma concentration-time curve of ligustilide after oral or intraperitoneal administration.

**Statistical Analysis** All data were expressed as means  $\pm$  standard deviation (S.D.). Unpaired student's *t*-test was used to compare results between two groups of animals receiving ligustilide intraperitoneally, while one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test was performed in order to compare results among different groups. Level of  $p < 0.05$  was considered significant.

## Results

**Validation of the HPLC Method** The HPLC method was demonstrated to be suitable for the quantification of ligustilide in samples collected from the *in vitro* and *in vivo* studies. Ligustilide was well separated from its metabolites (Fig. 1). The calibration curves for both plasma and incubated samples showed good linearity ( $r^2 > 0.999$  and  $r^2 > 0.996$ , respectively) over the concentration ranges tested. The overall intra- and inter-day variations were less than 5% (0.9%–4.2%) and recovery was higher than 87% (87.9%–99.7%), suggesting good reproducibility and precision. LOQ values for ligustilide were 20 ng/mL and 200 ng/mL in rat plasma and in Tris buffer, respectively.

**Pharmacokinetics of Ligustilide** Figure 2 shows plasma concentration-time curves of ligustilide after three routes of administration. The calculated pharmacokinetic parameters are summarized in Table 2. After intravenous administration, ligustilide exhibited extensive distribution in the body ( $V_d$ :  $3.76 \pm 1.23$  L/kg) and rapid elimination ( $t_{1/2}$ :  $0.31 \pm 0.12$  hr) from plasma. The plasma concentration decreased below LOQ after 1.5 hr (Fig. 2A). When a low dose of ligustilide (26 mg/kg) was given intraperitoneally (Fig. 2B), it was rapidly absorbed ( $T_{max}$ :  $0.05 \pm 0.02$  hr) and quickly eliminated ( $t_{1/2}$ :  $0.36 \pm 0.05$  hr). The intraperitoneal bioavailability was estimated to be 52%, indicating an extensive

hepatic first-pass metabolism. When a higher ip dose (52 mg/kg) of ligustilide was given, a markedly higher  $C_{\max}$  and a significantly lower CL/F were obtained compared with the lower ip dose; the absolute bioavailability was approximately 98%, suggesting non-linear and dose-dependent pharmacokinetics of ligustilide.

In the case of oral administration of ligustilide, plasma concentration-time profile and pharmacokinetic parameters could only be obtained at the highest dose (500 mg/kg). At lower doses, data were not meaningful because plasma levels of ligustilide were either below detectable limit (at 100 mg/kg) or they were not high enough for accurate pharmacokinetic analysis. At the dose of 500 mg/kg (Fig. 2C, Table 2), ligustilide was rapidly absorbed ( $T_{\max}$ :  $0.36 \pm 0.19$  hr) reaching a  $C_{\max}$  of  $0.66 \pm 0.23$   $\mu\text{g/mL}$ . Plasma concentration of ligustilide declined in a multi-phase manner. Levels dropped quickly to approximately 120 ng/mL within 4 hr and this level was maintained for another 4-8 hr before falling below LOQ. The longer lasting terminal phase (4-8 hr) significantly increased the retention of ligustilide (MRT:  $5.14 \pm 1.56$  hr) when compared with that of iv and ip data (Table 2). Values of CL/F were significantly higher and dose-normalized AUC lower than those obtained after iv and ip dosing. Oral bioavailability was estimated to be 2.6% at 500 mg/kg dose.

The elimination of ligustilide when administered in the form of Chuanxiong extract intravenously was significantly faster than that for its pure form (CL:  $20.35 \pm 3.05$  vs  $9.14 \pm 1.27$  L/hr/kg,  $p < 0.01$ , Fig. 2A, Table 2), suggesting interaction between ligustilide and components present in the extract.

**Identification of Metabolites of Ligustilide** Fifteen peaks (L1-L15) were found as potential metabolites *in vivo* (Fig. 1A-C). Among them, eight peaks (L1-2, L4-8 and L13)

were also found in the *in vitro* incubation studies with commercially available pure ligustilide (Fig. 1E). Formation of these eight metabolites required the presence of NADPH-regenerating system, indicating the involvement of NADPH-dependent monooxygenases. The spectra of seven out of eight metabolites, except L8, found in both *in vivo* and *in vitro* studies were obtained using the on-line HPLC-MS analysis (Fig. 3).

L1 and L2 exhibited similar mass spectra with protonated molecular ion ( $[M+H]^+$ ) and sodiated ion ( $[M+Na]^+$ ) at  $m/z$  225 and  $m/z$  247, respectively. In both spectra (Fig. 3), a diagnostic ion at  $m/z$  207 was found as the base peak, corresponding to the loss of a  $H_2O$  molecule ( $[M+H-H_2O]^+$ ), indicating primary/secondary hydroxyl substitution. Therefore, molecular weights of both metabolites were determined to be 224 Dalton, 34 mass units higher than that of ligustilide, indicating that comparing with the parent compound, two hydroxyl groups were added in the structures of these two metabolites. Furthermore, their retention time, UV and mass spectra were consistent with those of authentic senkyunolide I and senkyunolide H. These metabolites were generated from hydroxylation of ligustilide (Fig. 4). The mass spectrum of L13 showed the protonated molecular ion ( $[M+H]^+$ ) and a sodium adduct ion ( $[M+Na]^+$ ) at  $m/z$  189 and 211, respectively (Fig. 3). Its retention time, UV and mass spectra were identical to those of butylidenephthalide, which was formed via aromatization of ligustilide (Fig. 4). As indicated in Table 1, L1, L2 and L13 are present in Chuanxiong herb in relatively low quantities (Li et al, 2003; 2007; Yan et al, 2005).

The characteristic ions at  $m/z$  229 ( $[M+Na]^+$ ),  $m/z$  207 ( $[M+H]^+$ ) and  $m/z$  189 ( $[M+H-H_2O]^+$ ) in the mass spectrum of L7 (Fig. 3) indicated that this metabolite has a molecular weight of 206 Dalton, 16 mass units higher than L13, which corresponded to a monooxygenated metabolite of L13. Its retention time, UV and MS spectra were identical

to that of 3-hydroxybutylphthalide, a recently identified metabolite of butylidenephthalide in our laboratory (unpublished data). Therefore, L7 was identified as 3-hydroxybutylphthalide, it could be possibly generated from either direct hydration of ligustilide followed by aromatization or hydration of L13 as indicated in Fig. 4. The detailed information on the structure elucidation of 3-hydroxybutylphthalide will be published in a separate article on the metabolism of butylidenephthalide.

Structures of other three metabolites (L4-L6) were tentatively assigned. Mass spectrum of L4 (Fig. 3) exhibited the characteristic ions of  $[M+Na]^+$  at  $m/z$  229,  $[M+H]^+$  at  $m/z$  207, and  $[M+H-H_2O]^+$  at  $m/z$  189 as the base peak corresponding to the loss of a  $H_2O$  molecule from the primary/secondary hydroxyl group. The mass data demonstrated a molecular weight of 206 Dalton, 16 mass units higher than that of ligustilide, indicating a monooxygenated metabolite of ligustilide. Moreover, the UV spectrum of L4 was similar to that of ligustilide, suggesting the presence of an unchanged chromophore of the parent compound. Therefore, oxidation might occur at the end of 3-substituted side chain of ligustilide, and L4 was tentatively identified as 11-hydroxyligustilide. This metabolite was expected to be generated from direct oxidation of ligustilide (Fig. 4). L5 and L6 showed similar mass spectra with a protonated molecular ion ( $[M+H]^+$ ) at  $m/z$  514, which corresponded to the molecular weight of glutathione (GSH) conjugate of monohydroxylated ligustilide. The fragmentation ions at  $m/z$  385 and  $m/z$  207 represented characteristic ions due to the loss of glutamate ( $[M+H-Glutamate]^+$ ) and GSH ( $[M+H-GSH]^+$ ) moiety, respectively. Furthermore, the diagnostic ion due to the loss of both GSH and  $H_2O$  ( $[M+H-GSH-H_2O]^+$ ) at  $m/z$  189 indicated the presence of a hydroxyl group in both metabolites. Therefore, these two metabolites were assigned as isomers of glutathione

conjugate of monohydroxylated ligustilide (Fig. 4). However, the substituted positions for both GSH and hydroxyl group were unable to be unequivocally assigned. Based on the identified seven metabolites, the metabolic pathways of ligustilide in both *in vivo* and *in vitro* studies were proposed as shown in Fig. 4.

***In vitro* Metabolic Profiles** Ligustilide was neither metabolized in the rat intestinal S9 fractions nor microsomes. When pure ligustilide (300  $\mu$ M) was incubated with rat hepatic S9 preparations, approximately 60% ligustilide was metabolized in the presence of NADPH-regenerating system (Fig. 1D), but no significant metabolism was observed in the absence of NADPH-regenerating system (Fig. 1E). A similar metabolic pattern was found when ligustilide was incubated with hepatic microsomes. After ligustilide was incubated with hepatic microsomes in the presence of NADPH-regenerating system for 1 hr, over 98% of the substrate was metabolized. The most abundant metabolite generated was L1 (~21% of ligustilide), while L13 was identified as a minor metabolite (0.88% of ligustilide). The metabolic rate was rapid, significant saturation was not attained at concentration as high as 800  $\mu$ M (Fig. 5). However, due to solubility constraint, accurate Michaelis-Menton parameters, such as  $V_{\max}$  and  $K_m$ , for ligustilide could not be determined. The results indicated that ligustilide mainly underwent NADPH-dependent metabolism in the rat liver, which might be responsible for its extensive hepatic first-pass effect observed *in vivo*.

**Stability of Ligustilide in Simulated Gastric and Intestinal Fluids** Since no significant intestinal metabolism of ligustilide was observed, the stability of ligustilide in gastrointestinal tract was examined as a potential cause of low oral bioavailability. No significant degradation of ligustilide occurred in SGF and about 15 $\pm$ 8% degraded in SIF within 60 min (detailed data not shown).



## Discussion

Many medicinal herbs have a long history of clinical use. However, the safety and efficacy of most of these herbs in relation to their pharmacological activities are poorly understood. Furthermore, knowledge on the pharmacokinetics of the main bioactive ingredients of these herbs is extremely limited. Most of the data are acquired through studies which employed either purified/synthesized compound or herbal fractions/extracts. Studies comparing pure substrates and that present in an extract are rarely performed; therefore, potential interactions between the compounds of interest and other constituents in the extract are often not revealed.

Chuanxiong is one of the most commonly prescribed CM herbs for the treatment of cardiovascular diseases in China. However, pharmacokinetics of the main bioactive ingredients in this herb is largely unknown. There is only one published study on the oral pharmacokinetics of ligustilide (Shi et al., 2006). However, it is difficult to compare the reported results directly with our current findings, because the published protocol and sample preparations were not described accurately in detail. Moreover, oral bioavailability was not determined (Shi et al., 2006). In the present study, pharmacokinetics of ligustilide was investigated. The isolated ligustilide sample, which contained 5% butylidenephthalide with the composition ratio of two phthalides similar to that in the extract (approximately 18:1, Table 1), was utilized because 1) concentration of commercially available ligustilide was too low for *in vivo* dosing; and 2) pure ligustilide is light and thermally labile, but stable when kept in solvents, media, or mixed with other compounds (Cui et al., 2006; Li et al., 2007).

The results demonstrated that ligustilide was rapidly absorbed after ip and oral dosing and exhibited extensive distribution and rapid elimination after iv administration. The terminal phase after oral administration was significantly longer ( $p < 0.001$ ) than that of other two routes (Fig. 2). Given the oral bioavailability is less than 3%, the concentration achieved at the portal system during absorption was unlikely to be high enough to saturate hepatic enzymes; therefore nonlinear metabolism which leads to slower elimination is unlikely. However, the mechanism behind this observation is not clear. *In vitro* metabolic studies (Fig. 5) suggested that hepatic elimination could be quantitatively the most important route of elimination and partly contribute to the low oral bioavailability of ligustilide. On the other hand, the role of intestine leading to a low oral bioavailability is uncertain. Our previous study using Caco-2 cell model revealed high intestinal absorption of ligustilide with an absorptive permeability ( $P_{app}$ :  $4.19 \times 10^{-5}$  cm/sec) comparable with that of highly absorbable drugs (Ko et al., 2003), and the present *in vitro* studies demonstrated that the intestinal metabolism and degradation during the time frame of absorption are unlikely contributors to the extensive loss of ligustilide in the GI tract. Based on the data at hand, a plausible explanation is the rapid metabolism triggered by enzymes present in the intestinal lumen which include but not limited to that from intestinal flora. Moreover, lipophilic drugs are known to bind to tissues non-specifically; this process is extensive and saturable (Hussain, 1994). It is not clear whether ligustilide would extensively bind to intestinal and liver tissues. More studies are warranted.

Dose-dependent pharmacokinetics was observed after ip administration of ligustilide. The mechanisms responsible for this observation are not known. Saturation of metabolizing enzymes is unlikely because non-saturable metabolism were demonstrated at

concentration as high as 800  $\mu\text{M}$  *in vitro*. Furthermore, the elimination profiles after low and high ip dose were not significantly different from each other (Fig. 2B). The total body clearance value was much higher than that of hepatic blood flow, suggesting extrahepatic elimination is involved. However, it is not certain as to how extrahepatic elimination is involved. The disproportionate increase in AUC after a high dose of ligustilide may be due to saturation of non-specific binding in the liver. More study is required to elucidate this hypothesis.

In this study, constituents in Chuanxiong extract were found to significantly increase clearance of ligustilide (CL:  $20.35 \pm 3.05$  vs  $9.14 \pm 1.27$  L/hr/kg,  $p < 0.01$ ). The elimination of ligustilide is so high that it is likely to be flow limited. Since Chuanxiong and its products are used for the relief of pain induced by blood stagnation (China Pharmacopoeia, 2005), it is possible that the increase in ligustilide clearance may be a direct result of hepatic blood flow change. However, to date there is no published evidence supporting this contention. Interestingly, no significant pharmacokinetic interactions between senkyunolide A and components in Chuanxiong were found previously (Yan et al., 2007). Mechanisms responsible for pharmacokinetic interaction of ligustilide are unknown and worthy of investigation. The results revealed a variable and unpredictable pharmacokinetics of ligustilide, depending on components concurrently administered. Nevertheless, it is common to take more than one ingredient in herbal therapy. Therefore, therapeutic outcomes may vary when different Chuanxiong-based herbal preparations are utilized.

Eight ligustilide metabolites were identified both *in vivo* and *in vitro*. Butylidenephthalide, senkyunokide I, senkyunolide H and 3-hydroxybutylphthalide were unequivocally characterized; 11-hydroxyligustilide and two isomers of hydroxyligustilide

glutathione conjugate were tentatively identified, while structure of L8 was not elucidated. Several other peaks, which were only observed in chromatograms of plasmas after dosing but not in the incubated samples and blank plasma (Fig. 1), showed no ligustilide-related ions in their mass spectra and thus were not identified. The proposed metabolic pathways of ligustilide were reported for the first time (Fig. 4). All metabolites were formed via oxidation pathways mediated by NADPH-dependent monooxygenases, and two were subsequently converted to glutathione conjugates. Butylidenephthalide, senkyunokide I and senkyunolide H also occur naturally in Chuanxiong (Li et al., 2003; Yan et al., 2005), and have been reported to produce various pharmacological activities (Chan et al., 2006; Liang et al., 2005; Kobayashi et al., 1992). Being naturally present in herb and also as metabolites *in vivo*, it obviously make overall effects of all bioactive ingredients and their individual contributions in the body more complicated and difficult to be predicted. Therefore, it should be emphasized that quality control of the bioactive ingredients in Chuanxiong and its products must consider not only herbal contents but also quantities of their active metabolites *in vivo*.

Although to date there are no approved remedies containing ligustilide as a single active ingredient, large varieties of Chuanxiong-based proprietary products and herb itself, which all contain ligustilide as one of the most abundant constituents, are widely used in China and other communities worldwide. Although most of these products are taken orally in CM practice, the present findings for ligustilide together with a low oral bioavailability of senkyunolide A reported previously (Yan et al., 2007) provided important scientific data to challenge the validity of oral administration. The present study, in combination with our previous report on senkyunolide A, provided sound scientific evidence to support that

other administrative route, such as sublingual delivery, to avoid extensive first-pass effects in the gut and the liver might be more preferable. Furthermore, pharmacokinetic interactions between ligustilide and other components in the herb demonstrated that pharmacokinetic data obtained from a pure compound might be inadequate to predict its *in vivo* profile in herbal products.

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## Footnotes

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## Figure Legends

Fig. 1. HPLC chromatograms of rat blank plasma spiked with the isolated ligustilide sample (A), plasma obtained from rats dosed intravenously (B) or orally (C) with ligustilide, and rat liver S9 incubated with ligustilide in the absence (D) or presence (E) of NADPH-regenerating system

*L1: senkyunokide I, L2: senkyunolide H, L4: 11-hydroxylicustilide, L5 and L6: isomers of hydroxylicustilide glutathione conjugates, L7: 3-hydroxybutylphthalide, and L13: butylidenephthalide, L3, L8-12 and L14-L15: unidentified peaks, IS: Internal Standard*

Fig. 2. Mean plasma concentration-time profiles of ligustilide in rats after intravenous (A), intraperitoneal (B) and oral (C) administration (n=5).

*\*Data were obtained from one rat*

Fig. 3. Mass and UV spectra of metabolites L1, L2, L4-L7 and L13 obtained by HPLC-UV-MS analysis

*L1: senkyunokide I, L2: senkyunolide H, L4: 11-hydroxylicustilide, L5 and L6: isomers of hydroxylicustilide glutathione conjugates, L7: 3-hydroxybutylphthalide, and L13: butylidenephthalide.*

Fig. 4. The proposed metabolic pathways of ligustilide in the rat

*The unidentified intermediate is shown in the large bracket. Gly: glycine; Cys: cysteine; Glu: glutamate*

Fig. 5. Velocity of the loss of liquistilide in the hepatic microsomal incubations in the presence of NADPH-regenerating system (n=3)

Table 1. Content of main phthalides in Chuanxiong extract

Phthalide	Content (% , w/w)
Ligustilide	14.9
Butylidenephthalide	0.84
Senkyunolide A	7.65
Senkyunolide I	1.17
Senkyunolide H	0.38
Neoclidilide	0.19
Tokinolide B	0.20
Levistolide A	0.82

Table 2. Pharmacokinetic parameters of ligustilide in rats after intravenous (iv), intraperitoneal (ip) and oral administration (n=5)

Pharmacokinetic parameter	Administration route				
	iv		ip		oral
Dose (mg/kg)	15.6	14.9 <sup>a</sup>	26	52	500
T <sub>max</sub> (hr)	-	-	0.05±0.02	0.08±0.01	0.36±0.19
C <sub>max</sub> (mg/L)	13.19±0.84	6.93±0.60 <sup>***</sup>	7.48±1.10 <sup>***</sup>	20.75±2.55 <sup>###</sup>	0.66±0.23 <sup>***</sup>
t <sub>1/2</sub> (hr)	0.31±0.12	0.22±0.07	0.36±0.05	0.44±0.08 <sup>#</sup>	3.43±1.01 <sup>***</sup>
AUC <sub>0-∞</sub> (mg•hr/L) <sup>b</sup>	1.81±0.24	0.79±0.10 <sup>**</sup>	0.93±0.07 <sup>*</sup>	1.77±0.23 <sup>#</sup>	0.047±0.012 <sup>**</sup>
V <sub>d</sub> /F(L/kg) <sup>ϕ</sup>	3.76±1.23	5.62±1.19	6.54±1.56	6.32±1.81	1641.9±121.6 <sup>***</sup>
CL/F (L/hr/kg) <sup>ϕ</sup>	9.14±1.27	20.35±3.05 <sup>**</sup>	16.90±1.21 <sup>**</sup>	9.26±1.04 <sup>##</sup>	411.1±145.7 <sup>***</sup>
MRT (hr)	0.30±0.07	0.19±0.03	0.30±0.05	0.41±0.03	5.14±1.56 <sup>***</sup>
F (%)	-	45.7 <sup>!</sup>	51.7	97.7	2.6

<sup>a</sup>Dose of ligustilide in 100 mg/kg of Chuanxiong extract

<sup>b</sup>Normalized with dose

<sup>ϕ</sup>Data represent V<sub>d</sub> and CL in the case of iv dosing of the isolated ligustilide

<sup>!</sup>Relative bioavailability compared with that of iv dosing of the isolated ligustilide

<sup>\*</sup>p < 0.05, <sup>\*\*</sup>p < 0.01, <sup>\*\*\*</sup>p < 0.001, compared with iv dosing of the isolated ligustilide

<sup>#</sup>p < 0.05, <sup>##</sup>p < 0.01, <sup>###</sup>p < 0.01, compared with the lower ip dose of the isolated ligustilide

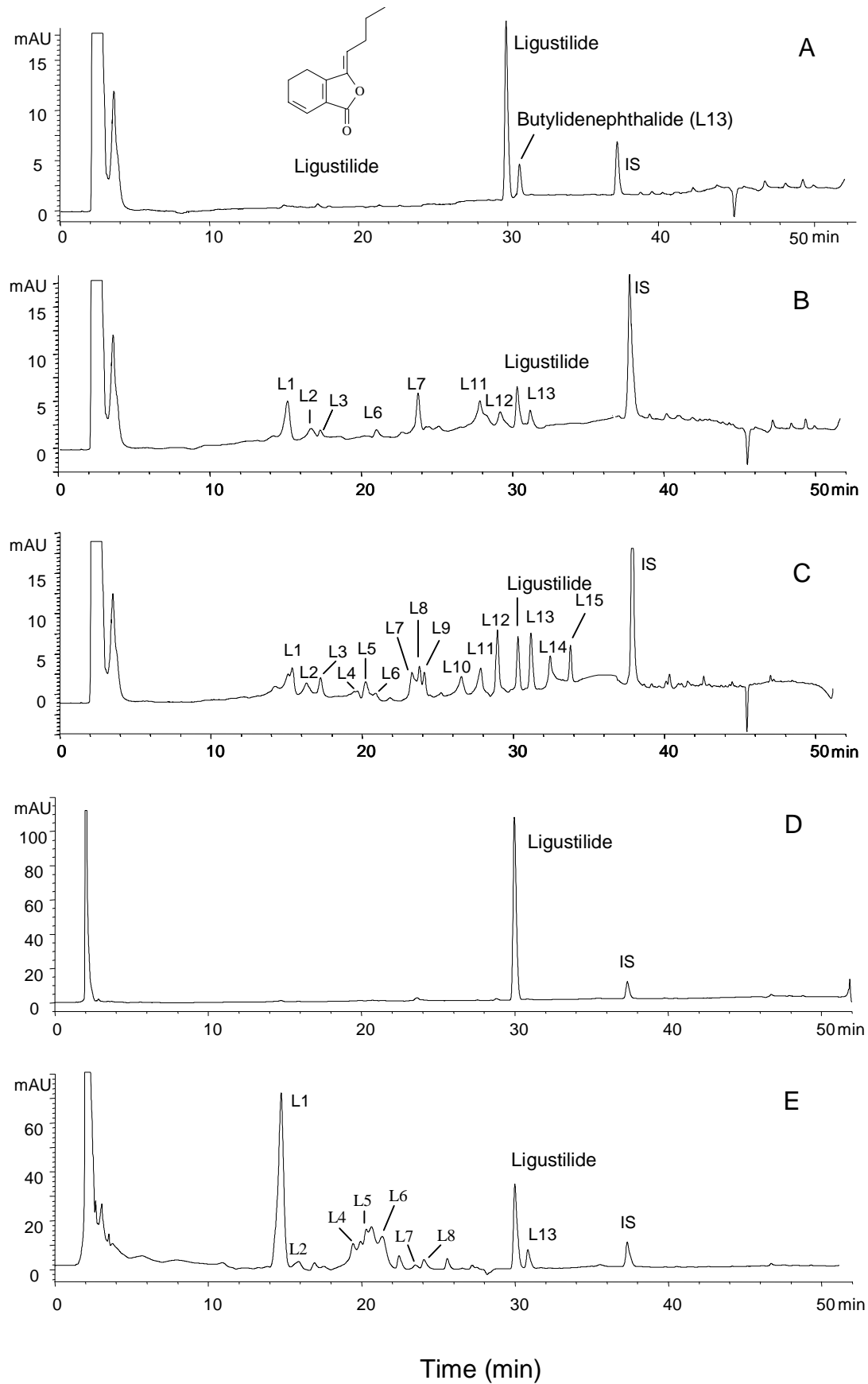


Fig. 1

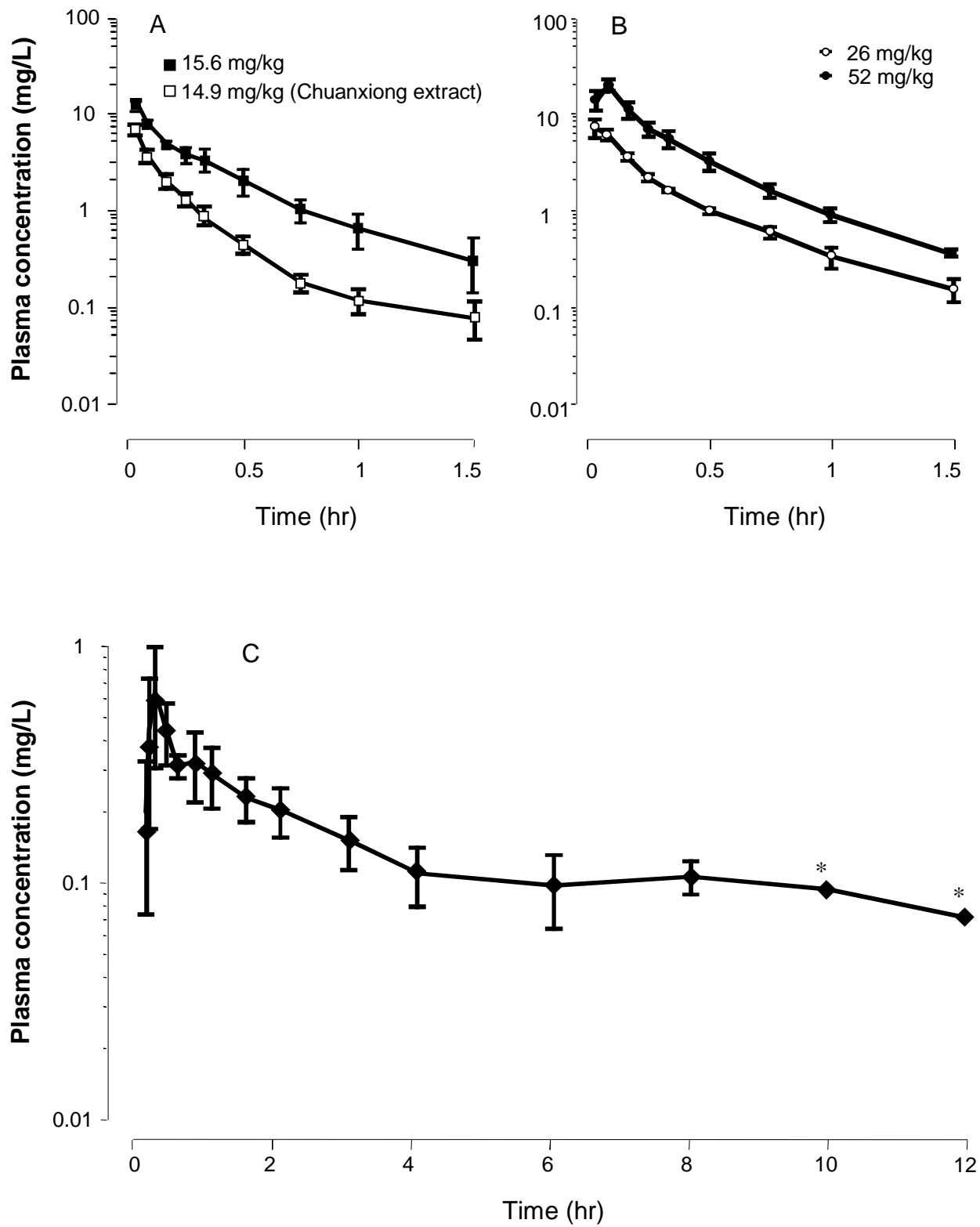


Fig. 2



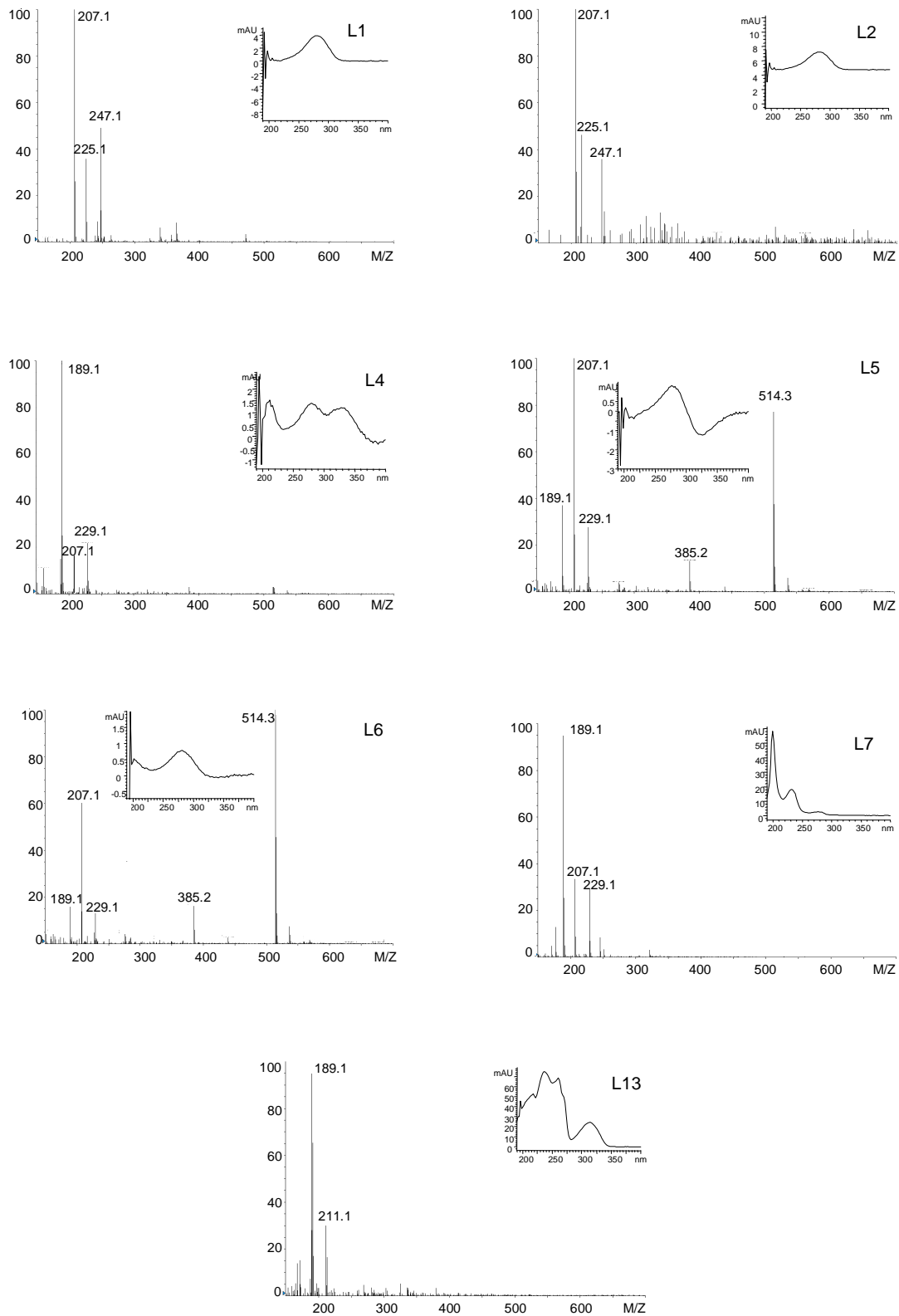


Fig. 3

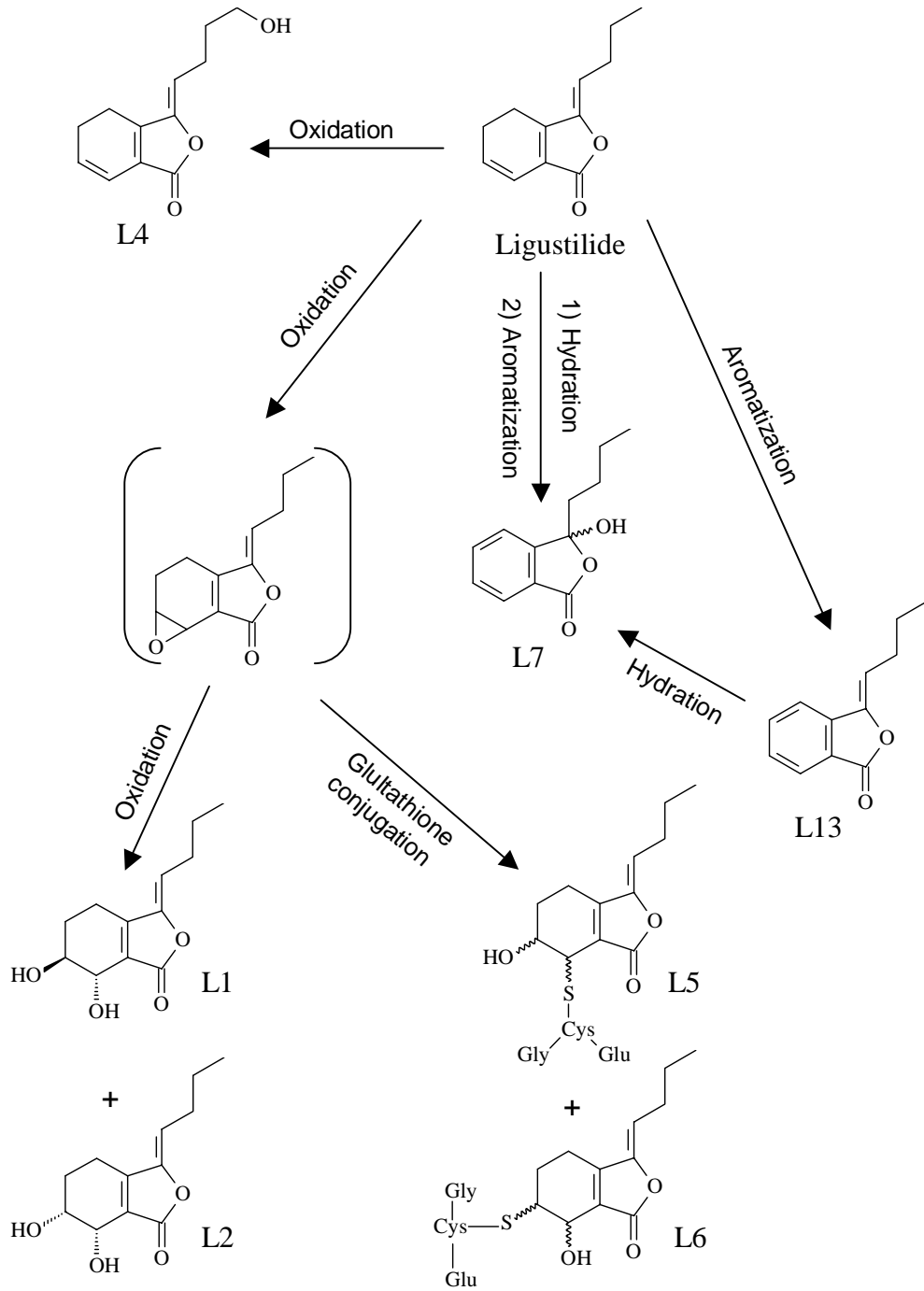


Fig. 4

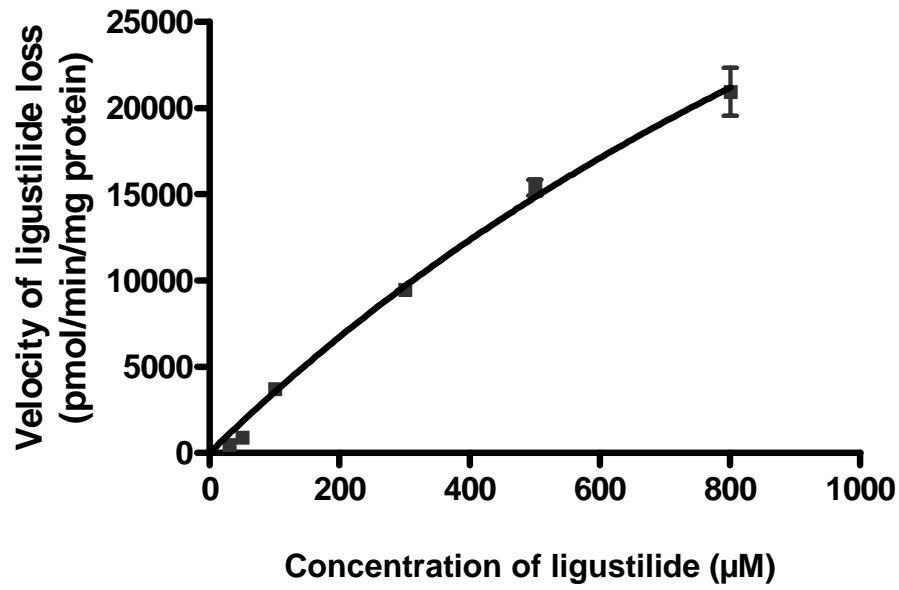


Fig. 5