Pharmacokinetics and Metabolism of Ligustilide, a Major Bioactive Component in Rhizoma Chuanxiong, in the Rat

Ru Yan, Nga Ling Ko, Song-Lin Li, Yun Kau Tam, and Ge Lin

Department of Pharmacology, Faculty of Medicine, the Chinese University of Hong Kong, Shatin, N.T., Hong Kong, SAR (R.Y., N.L.K., S.-L.L., G.L.); and Sinoveda Canada Inc., Edmonton, Alberta, Canada (Y.K.T.)

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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 i.v. administration of pure ligustilide, it was distributed extensively ($V_{d} = 3.76 \pm 1.23 \text{ l/kg}$) and eliminated rapidly ($t_{1/2} = 0.31 \pm 0.12 \text{ h}$). The i.v. clearance (CL) of ligustilide after Chuanxiong extract administration was significantly higher than that dosed in its pure form [CL, $20.35 \pm 3.05 \text{ versus } 9.14 \pm 1.27 \text{ l/h/kg}$, $p < 0.01$; area under the curve (AUC), $0.79 \pm 0.10 \text{ versus } 1.81 \pm 0.24 \text{ mg h/l}$, $p < 0.01$], suggesting significant interaction between ligustilide and components present in the extract. Dose-dependent pharmacokinetics was observed after i.p. administration, and a significantly higher dose-normalized AUC ($1.77 \pm 0.23 \text{ mg h/l}$) at 52 mg/kg was obtained than that at 26 mg/kg ($0.93 \pm 0.07 \text{ mg h/l}$, $p < 0.05$). Oral bioavailability of ligustilide was low (2.6%), which was partly because of 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 (State Pharmacopoeia Commission of P.R. China, 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 (Sun et al., 2002; Li, 2005).

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; Ko et al., 1997, 1998, 2002; Matsumoto et al., 1998; 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 (Naito et al., 1995; Ko et al., 1998, 2002; Matsumoto et al., 1998; Chong and Feng, 1999; Cao et al., 2006; Chan et al., 2006, 2007; Du et al., 2006; Lu et al., 2006). Ligustilide (Z-3-butyldiene-4,5-dihydroisodenzofuranone) (Fig. 1), a phthalide derivative, is the most abundant constituent in the herb (Li et al., 2002, 2006; Yan et al., 2005). Vasodilatation, antiplatelet aggregation, antithrombotic, serotoninergic activity, and antiproliferative properties of ligustilide have been well documented (Naito et al., 1995; Cao et al., 2006; Deng et al., 2006; Du et al., 2006; Lu et al., 2006; Chan et al., 2007). Hence, ligustilide is considered to be a key active ingredient in Chuanxiong.

Like many medicinal herbs, most Chuanxiong products are taken p.o. 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 p.o. 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 p.o. 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.

ABBREVIATIONS: CM, Chinese medicine; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SD, Sprague-Dawley; LOQ, limit of quantification; AUC$_{0-\infty}$, area under the plasma concentration-time curve from time 0 to time infinity; MRT, mean residence time; GSH, glutathione.
FIG. 1. HPLC chromatograms of rat blank plasma spiked with the isolated ligustilide sample (A), plasma obtained from rats dosed i.v. (B) or p.o. (C) with ligustilide, and rat liver S9 incubated with ligustilide in the absence (D) or presence (E) of NADPH-regenerating system. L1, senkyunolide I; L2, senkyunolide H; L4, 11-hydroxyligustilide; L5 and L6, isomers of hydroxyligustilide glutathione conjugates; L7, 3-hydroxybutylphthalide; and L13, butylidenephthalide; L3, L8–L12, and L14–L15, unidentified peaks; IS, internal standard.
Furthermore, in vitro studies were conducted to study ligustilide metabolism in greater detail.

### Materials and Methods

**Chemicals.** Methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Loughborough, Leicestershire, UK). Z-Ligustilide (ligustilide, 99% pure, structure shown in Fig. 1) in dimethyl sulfoxide (10 mg/ml) was purchased from ChromaDex (Irvine, CA), whereas n-butylidenephthalide (butylidenephthalide, 96% pure) and all the other chemicals were from Sigma (St. Louis, MO). Chuanxiong crude oil extract (Chuanxiong extract) prepared using CO₂ 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. Therefore, this preparation was used 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). Because of the instability of ligustilide in its pure form as reported previously (Lin et al., 1998; Cui et al., 2006), the chromatographically “purified” ligustilide also contained 5% of butylidenephthalide. Identities of all the isolated compounds were confirmed by matching their retention times, UV, and mass spectrometry (MS) spectra with those of the authentic standards using our previously developed HPLC/UVMS 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 (five animals in each group). For animals receiving i.v. dosing, a femoral vein cannula was also implanted. After cannulation, rats were allowed to recover and fasted overnight with free access to water. For i.v. administration, the “purified” ligustilide sample (ligustilide dose, 15.6 mg/kg) or Chuanxiong extract (ligustilide dose, 14.9 mg/kg) formulated in Pharmatek formulation-6 (Pharmatek, San Diego, CA) 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 p.o. (100, 360, or 500 mg/kg) or i.p. (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-h period as illustrated in Fig. 2. After each collection, 0.3 ml of saline containing 40 IU of heparin/ml was injected to compensate for blood loss. Blood samples were centrifuged at 3000g for 10 min, and plasma samples were harvested. Each plasma sample (160 μl) was mixed with 5 μl of internal standard (α-naphthoflavone, 40 μg/ml) and 165 μl of acetonitrile, vortexed, and centrifuged at 16,000g for 5 min. The supernatant was filtered with a 0.45-μm syringe filter, and 100 μl of the filtrate was subjected to HPLC analysis.

### In Vivo Metabolic Study.

Pooled (five untreated male SD rats) liver and small intestine homogenates (S9) and microsomes were prepared using standard methods (Williams et al., 1989; Lin et al., 2000, 2007). Total contents of protein and cytochrome P450 were measured using the Lowry (Lowry et al., 1951) and the Omura methods (Omura and Sato, 1964), respectively. The reaction components contained one of the subcellular preparations (2 mg of protein/ml) described above, ligustilide (5–800 μM), NADPH-regenerating system (1 mM NADP⁺, 1 mM NAD⁺, 10 mM glucose 6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 4 mM MgCl₂), 50 mM Tris buffer containing 150 mM 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 h 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 and filtered, and 100 μl of filtrate was subjected to HPLC analysis. All the controls and reactions were 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 containing no pepsin (0.05 M sodium chloride adjusted to pH 1.5 using HCl) (The United States Pharmacopeial Convention, 2006) or simulated intestinal fluid containing no pancreatin (0.05 M sodium dihydrogen phosphate buffer adjusted to pH 6.8 using NaOH) (The United States Pharmacopeial Convention, 2006). The mixtures were incubated for 1 h at 37°C. Aliquots (100 μl) of incubates were collected at 0, 5, 15, 30, and 60 min. Each aliquot was immediately extracted with ethyl acetate (200 μl), followed by centrifugation at 500g 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 μl) was mixed with 150 μl of methanol containing 1.75 μg of α-naphthoflavone. The resultant solution was filtered, and an aliquot (100 μl) 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/α-naphthoflavone) of the samples with that at time 0.

**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/UVMS method as described below.

The chromatographic condition was the same as described under Quantitative Analysis. The online HPLC/UVMS analysis was performed on a PerkinElmer series 200 liquid chromatograph connected to a PerkinElmer Series 200 triple-quadrupole mass selective mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) equipped with electrospray ionization interface. Positive electrospray ionization/MS was conducted under the following conditions: nebulizing gas, 40 psi; auxiliary gas, 60 psi; curtain gas, 30 psi; turbo-ion spray 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 through 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/diode array detector (Agilent Technologies, Palo Alto, CA) and a Waters (Milford, MA) Symmetry C18 column (5 μm, 150 × 4.6 mm) coupled with a guard column (Waters Spherisorb S5 ODS2, 10 × 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 a wavelength of 284 nm.

**Calibration curves of ligustilide.** A methanol stock solution of ligustilide was diluted to the desired concentrations with methanol. Aliquot (5 μl) of each diluted solution was spiked into pooled plasma (three untreated male SD rats) to give concentrations ranging from 0.2 to 25 mg/l (0.2, 0.8, 2, 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.

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**TABLE 1**

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<tr>
<th>Phthalide</th>
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<tr>
<td>Butylidenephthalide</td>
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<tr>
<td>Senkyunolide A</td>
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<tr>
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<tr>
<td>Senkyunolide H</td>
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<td>Neocladilide</td>
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<td>Tokinolide B</td>
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<tr>
<td>Levistilide A</td>
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thoroughly, then treated and analyzed in the same manner as described under 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 intraday and interday variability. Samples at each concentration were analyzed in triplicates to examine intraday variability. The interday variability was determined on three separate days over a period of 1 week. Peak area ratio for each concentration was measured, and concentration of ligustilide was calculated from the corresponding calibration curve. Relative standard deviation 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 noncompartmental analysis (WinNonlin 4.0, Pharsight, Mountain View, CA). The area under the plasma concentration-time curve from time 0 to time infinity (AUC_{0\rightarrow\infty}) was calculated using the linear/log trapezoidal rule-extrapolation method, in which the linear trapezoidal rule was used for the calculation of the area during the ascending phase with the logarithmic trapezoidal rule for the declining phase. C_{\text{max}} and T_{\text{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:

\[
\text{CL/F} = \frac{\text{Dose}}{\text{AUC}_{0\rightarrow\infty}}
\]

\[
\text{V}_{d}/F = \frac{\text{Dose} \times (\lambda z \times \text{AUC}_{0\rightarrow\infty})}{\text{CL/F}}
\]

\[
\text{MRT} = \frac{\text{AUC}_{0\rightarrow\infty}}{\text{CL/F} \times \text{Dose}}
\]

\[
\text{F} = \frac{(\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{oral}}) + (\text{AUC}_{\text{i.v.}} \times \text{Dose}_{\text{i.v.}})}{(\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{oral}}) + (\text{AUC}_{\text{i.v.}} \times \text{Dose}_{\text{i.v.}})}
\]

where \(\lambda z\) is the terminal rate constant; AUC_{0\rightarrow\infty} is the area under the first moment curve from time 0 to infinity; Dose_{oral} is the i.v. dose of ligustilide; and Dose_{p.o./i.p.} is the p.o. or i.p. dose of ligustilide. AUC_{i.v.} is the area under the plasma concentration-time curve of ligustilide after i.v. administration; AUC_{p.o./i.p.} is the corresponding area under the plasma concentration-time curve of ligustilide after p.o. or i.p. administration.

FIG. 2. Mean plasma concentration-time profiles of ligustilide in rats after i.v. (A), i.p. (B), and p.o. (C) administration (n = 5). * Data were obtained from one rat.
Pharmacokinetic parameters of ligustilide in rats after i.v., i.p., and p.o. administration (n = 5)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Administration Route</th>
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<tr>
<td></td>
<td>i.v.</td>
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<tr>
<td>T_{1/2} (h)</td>
<td></td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>13.19 ± 0.84</td>
</tr>
<tr>
<td>T_{1/2} (h)</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng/ml h)</td>
<td>1.81 ± 0.24</td>
</tr>
<tr>
<td>V_d/F (l/kg)</td>
<td>3.76 ± 1.23</td>
</tr>
<tr>
<td>CL/F (l/h/kg)</td>
<td>9.14 ± 1.27</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>F (%)</td>
<td>45.7^d</td>
</tr>
</tbody>
</table>

* p < 0.05, **p < 0.01, ***p < 0.001, compared with i.v. dosing of the isolated ligustilide.
† p < 0.05, **p < 0.01, ***p < 0.001, compared with the lower i.p. dose of the isolated ligustilide.
‡ Dose of ligustilide in 100 mg/kg of Chuanxiong extract.
§ Normalized with dose.
Data represent V_d and CL in the case of i.v. dosing of the isolated ligustilide.
Relative bioavailability compared with that of i.v. dosing of the isolated ligustilide.

### Results

#### Validation of the HPLC Method
The HPLC method was shown 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 intraday and interday 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 and 200 ng/ml in rat plasma and 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 i.v. administration, ligustilide exhibited extensive distribution in the body (V_d, 3.76 ± 1.23 l/kg) and rapid elimination (T_{1/2}, 0.31 ± 0.12 h) from plasma. The plasma concentration decreased below LOQ after 1.5 h (Fig. 2A). When a low dose of ligustilide (26 mg/kg) was given i.p. (Fig. 2B), it was rapidly absorbed (T_{max} 0.05 ± 0.02 h) and quickly eliminated (T_{1/2}, 0.36 ± 0.05 h). The i.p. bioavailability was estimated to be 52%, indicating an extensive hepatic first-pass metabolism. When a higher i.p. 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 i.p. dose; the absolute bioavailability was approximately 98%, suggesting nonlinear and dose-dependent pharmacokinetics of ligustilide.

In the case of p.o. 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 ± 0.19 h) reaching a C_{max} of 0.66 ± 0.23 mg/ml. Plasma concentration of ligustilide declined in a multiphasic manner. Levels decreased quickly to approximately 120 mg/ml within 4 h, and this level was maintained for another 4 to 8 h before decreasing below LOQ. The longer-lasting terminal phase (4–8 h) significantly increased the retention of ligustilide (MRT, 5.14 ± 1.56 h) when compared with that of i.v. and i.p. data (Table 2). Values of CL/F were significantly higher and dose-normalized AUC lower than those obtained after i.v. and i.p. dosing. Oral bioavailability was estimated to be 2.6% at the 500-mg/kg dose.

The elimination of ligustilide when administered in the form of Chuanxiong extract i.v. was significantly faster than that for its pure form (CL, 20.35 ± 3.05 versus 9.14 ± 1.27 l/h/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. 1, A–C). Among them, eight peaks (L1–L2, L4–L8, 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 monoxygenases. The spectra of seven of eight metabolites, except L8, found in both in vivo and in vitro studies were obtained using the online 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 247, respectively. In both spectra (Fig. 3), a diagnostic ion at m/z 229 ([M+Na]^+) was found as the base peak, corresponding to the loss of an H2O molecule ([M+H2O]^+), indicating primary/secondary hydroxyl substitution. Therefore, molecular masses of both metabolites were determined to be 224 Da, 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+H2O]^+) in the mass spectrum of L7 (Fig. 3) indicated that this metabolite has a molecular mass of 206 Da, 16 mass units higher than L13, which corresponds to a monoxygenated metabolite of L13. Its retention time, UV, and MS spectra were identical to that of 3-hydroxybutylphthalide, a recently identified...
Fig. 3. Mass and UV spectra of metabolites L1, L2, L4–L7, and L13 obtained by HPLC/UV/MS analysis. L1, senkyunolide I; L2, senkyunolide H; L4, 11-hydroxyligustilide; L5 and L6, isomers of hydroxyligustilide glutathione conjugates; L7, 3-hydroxybutylphthalide; L13, butylidenephthalide.
metabolite of butylidenephthalide in our laboratory (unpublished data). Therefore, L7 was identified as 3-hydroxybutylphthalide, and 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-H₂O]⁺ at m/z 189 as the base peak corresponding to the loss of an H₂O molecule from the primary/secondary hydroxyl group. The mass data showed a molecular mass of 206 Da, 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 mass of glutathione (GSH) conjugate of monohydroxylated ligustilide. The fragmentation ions at m/z 385 and 207 represented characteristic ions as a result of the loss of glutamate ([M+H-Glutamate]⁺) and GSH ([M+H-GSH]⁺) moiety, respectively. Furthermore, the diagnostic ion resulting from the loss of both GSH and H₂O ([M+H-GSH-H₂O]⁺) 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 metabolized neither in the rat intestinal S9 fractions nor microsomes. When pure ligustilide (300 μM) 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 h, more than 98% of the substrate was metabolized. The
most abundant metabolite generated was L1 (~21% of ligustilide), whereas L13 was identified as a minor metabolite (0.88% of ligustilide). The metabolic rate was rapid, and significant saturation was not attained at concentrations as high as 800 µM (Fig. 5). However, because of solubility constraint, accurate Michaelis-Menten parameters, such as $V_{\text{max}}$ 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. Because 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 simulated gastric fluid and about 15 ± 8% degraded in simulated intestinal fluid 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 of the pharmacokinetics of the main bioactive ingredients of these herbs is extremely limited. Most of the data are acquired through studies that used 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 used 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 showed that ligustilide was rapidly absorbed after i.p. and p.o. dosing and exhibited extensive distribution and rapid elimination after i.v. administration. The terminal phase after p.o. 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 that 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 the Caco-2 cell model revealed high intestinal absorption of ligustilide with an absorptive permeability ($P_{\text{app}}$, 4.19 × 10⁻⁵ cm/s) comparable with that of highly absorbable drugs (Ko et al., 2003), and the present in vitro studies showed that the intestinal metabolism and degradation during the time frame of absorption are unlikely contributors to the extensive loss of ligustilide in the gastrointestinal 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 are not limited to those from intestinal flora. Moreover, lipophilic drugs are known to bind to tissues nonspecifically; this process is extensive and saturable (Hussain et al., 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 i.p. administration of ligustilide. The mechanisms responsible for this observation are not known. Saturation of metabolizing enzymes is unlikely because nonsaturable metabolism was shown at concentrations as high as 800 µM in vitro. Furthermore, the elimination profiles after low and high i.p. 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 because of saturation of nonspecific 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 ± 3.05 versus 9.14 ± 1.27 l/h/kg; $p < 0.01$). The elimination of ligustilide is so high that it is likely to be flow-limited. Because Chuanxiong and its products are used for the relief of pain induced by blood stagnation (State Pharmacopoeia Commission of P.R. China, 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 used.

Eight ligustilide metabolites were identified both in vivo and in vitro. Butylidenephthalide, senkyunolide I, senkyunolide H, and 3-hydroxybutylphthalide were unequivocally characterized; 11-hydroxyligustilide and two isomers of hydroxyligustilide glutathione conjugate were tentatively identified, whereas 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 the metabolites were formed via oxidation pathways mediated by NADPH-dependent monoxygenases, and two were subsequently converted to glutathione conjugates. Butylenephthalide, senkyunolide 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 effects (Kobayashi et al., 1992; Liang et al., 2005; Chan et al., 2006). Being naturally present in herbs and also as metabolites in vivo, it obviously makes overall effects of all the 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 p.o. 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 p.o. administration. The present study, in combination with our previous report on senkyunolide A, provided sound scientific evidence to support that another 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 herbs showed that pharmacokinetic data obtained from a pure compound might be inadequate to predict its in vivo profile in herbal products.

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References

Address correspondence to: Ge Lin, Department of Pharmacology, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR. E-mail: linge@ cuhk.edu.hk.