Effect of co-existent components in CO$_2$ supercritical fluid extract of Angelica Sinensis Radix on metabolism of Z-ligustilide after oral administration in rats

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KEYWORDS
Angelica Sinensis Radix;
Supercritical fluid extract;
Z-ligustilide;
Metabolism

Abstract  Objective: To establish a basis for Angelica Sinensis Radix (ASR) as a dietary supplement for colorectal cancer chemoprevention, the effect of co-existent components in supercritical fluid extract (SFE) of ASR on the pharmacokinetics of Z-ligustilide after oral administration was investigated in vitro and in vivo.

Methods: Incubation in gastrointestinal contents and incubation in rat liver tissue homogenates post-mitochondrial supernatant (PMS) experiments were used to study changes in the levels of Z-ligustilide in vitro.

Results: Within 4 hours, the level of Z-ligustilide in SFE declined at a slower rate than in its pure form. Clearance of Z-ligustilide after administration in its pure form was significantly slower than that of SFE of ASR (CL, 0.96 ± 0.16 mL·min/kg versus 1.24 ± 0.21 mL·min/kg $P < 0.05$; AUC, 243.37 ± 16.84 versus 176.69 ± 12.59 mg·min/L).

Conclusion: These phenomena may be attributed to the interactions between the co-existent components in SFE of ASR and Z-ligustilide enhancing the stability of Z-ligustilide. These results suggest that the bioavailability of Z-ligustilide in SFE of ASR is improved. However, stabilization of plasma concentration was not sustained, so that the efficacy of active components could not be maintained. Thus, further processing of SFE of ASR is required.

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Introduction

The Chinese herb Angelica Sinensis Radix (ASR) is the dry root of Angelica sinensis (Oliv.) Diels. The Chinese name of ASR is dang gui (当归), and the herb has been used as a traditional Chinese medicine for nearly two thousand years. Traditional Chinese medicine theory holds that ASR promotes blood circulation, tonifies the blood, relieves pain, and moistens the bowel. Modern pharmacologic research has shown that ASR has anti-coagulation and anti-inflammatory properties. In addition to its medicinal use, ASR is a common ingredient in home cooking in China.

Supercritical fluid extraction (SFE) is a method used to separate out, or extract, chemical compounds. Supercritical Fluid Extraction is the process of separating or extracting the chemical compounds from the matrix using supercritical fluids as the extracting solvent. Carbon dioxide is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. SFE is applied in several fields, including the petroleum, cosmetic, food, pharmaceutical, and nutraceutical industries. CO2 at or above its critical temperature and critical pressure is in the state of fluid and is known as supercritical CO2. Supercritical CO2 is a common solvent used in chemical extractions because of its stability, permeability, and solubility. As extraction and separation are carried out at a low temperature, decomposition and volatilization of the extracted compound are prevented. Thus, safety, efficiency, economy, and environmental protection are the hallmarks of CO2 supercritical fluid extraction.

In previous studies, we identified the process parameters of CO2 SFE of ASR. Chemical composition of ASR was characterized by gas chromatography-mass spectrometry. Based on our research, Z-ligustilide is the most abundant active component in ASR, reaching a proportion of 42.12% ± 1.78% (m/m). Z-ligustilide was first extracted from Ligusticum acutilobum Siebold & Zucc. by Mitsuhashi in 1960. L acutilobum Siebold & Zucc. is also known as Angelica acutiloba (Siebold & Zucc.) Kitag, which is in the same genus as Angelica sinensis (Oliv.) Diels., or ASR. Research on the pharmacologic activities of Z-ligustilide has discovered that it has an anti-oxidative property and induces vasodilation, thus improving microcirculation. Z-ligustilide has also been found to activate Nrf-2/ARE-mediated gene expression and induce anti-inflammatory activities directly through epigallocatechin gallate (EGCG). We have also reported that SFE of ASR exhibited colorectal cancer preventive potential in azoxymethane/dextran sodium sulfate (AOM/DSS) mice model through activation of the Nrf-2/ARE pathway. However, results indicated that even though SFE of ASR could decrease tumor incidence, high/low-grade intraepithelial neoplasia was still present at approximately 50%. Further investigation is required to assure whether the route of administration limited the work of Z-ligustilide.

The gastrointestinal tract of animals is a complex environment that contains digestive enzymes and microorganisms. This affects the stability and metabolism of drugs in the digestive tract. Thus, to study the effect of the gastric and intestinal environment on the metabolism of Z-ligustilide, the gastrointestinal environment was simulated by dissolving the contents of digestive track using artificial digestion. Additionally, as the liver is a major organ of metabolism, liver tissue homogenate post-mitochondrial supernatant (PMS) is a rapid and easy technique to study drug decomposition.

This study was conducted to compare changes in Z-ligustilide level in gastrointestinal contents and in liver tissue homogenate PMS in its pure form and SFE of ASR. Pharmacokinetics of Z-ligustilide in its pure form and SFE of ASR were first compared. Interaction between Z-ligustilide and co-existent components in SFE of ASR were then explored.

Materials and methods

Chemicals

Angelica Sinensis Radix was purchased from Ben Cao Fang Yuan Medical Materials (Beijing, China; No.17643960) and identified by Professor Chunsheng Liu (Beijing University of Chinese Medicine). SFE of ASR was extracted as described previously. Z-ligustilide was separated from SFE of ASR by silica gel column chromatography developed in our laboratory, and reached purity of more than 95%.

Methanol for high-performance liquid chromatography (HPLC) was purchased from Fisher Scientific Co. (Shanghai, China), and internal standard, α-naphthoflavone was purchased from Sigma–Aldrich Co. (Shanghai, China). All other reagents were of analytical grade, and purchased from Sinopharm Chemical Reagent Co. (Beijing, China).

Experimental animals

Male Sprague–Dawley (SD) rats (220 ± 10 g) were supplied by SPF Experimental Animal Science and Technology Ltd. (Beijing, China). Animals were housed in the Beijing University of Chinese Medicine Laboratory Animal Department under standard conditions of temperature, humidity, and light. Laboratory mice and rats formula feeds and water were provided ad libitum.

In vitro protocol

Preparation of rat gastrointestinal contents

Male SD rats (n = 6), that were allowed to fast 12 hours before the experiment were sacrificed by cervical vertebra dislocation. The stomach, from cardia to pylorus, was removed and dissected. Its contents were immersed in 5 mL artificial gastric juice, and the gastric mucosa was eluted repeatedly with another 5 mL artificial gastric juice. The gastric contents were obtained by pooling 10 mL of the artificial gastric juice.

Intestine, from 10 cm below the pylorus, was sectioned at 30 cm and dissected. Artificial intestinal juice was applied with the same manner as gastric content preparation.

Incubation of ligustilide in gastrointestinal contents

SFE of ASR and Z-ligustilide emulsions were prepared by first weighing 71.23 mg SFE of ASR and 30.00 mg Z-ligu-
Stillide and then adding 1.00% (0.10 g) Tween-80. After thoroughly blending, 10 mL distilled water was dropped gradually with stirring.

Ten milliliters gastric or intestinal contents were mixed with 100 μL SFE or Z-ligustilide emulsion in a beaker, which was covered by aluminum foil to protect it from light. The beaker was placed on a thermostatic magnetic stirrer. Temperature was set to 37°C, and stirring speed to 180 rpm. At 2-h intervals, 100 μL samples were collected. After each collection, 10 μL internal standard and 190 μL methanol were added to the sample, which was then vortexed for 1 minute followed by centrifuging at 16 000 g for 10 minutes. Finally, 10 μL supernatant was harvested for HPLC analysis. Each group’s experiment was performed in parallel 6 times.

Preparation of rat liver tissue homogenates PMS

Male SD rats (n = 6) that were allowed to fast 12 hours before the experiment were sacrificed by cervical vertebra dislocation. Whole liver was harvested and liver tissue was weighed, and immersed 4 times in 0.25 mol/L sucrose solution. The liver tissue was homogenized over an ice bath until there were no visible blocks of tissue. After centrifuging at 12 000 g for 15 minutes and removing the sediment, the liver tissue homogenate post-mitochondrial supernatant (PMS), containing liver hepatosome and cytoplasm, was obtained.13

Incubation of ligustilide in gastrointestinal contents

Ten milliliters liver tissue PMS were mixed with 100 μL SFE or Z-ligustilide emulsion in a beaker, which was covered by aluminum foil to protect it from light. The beaker was placed on a thermostatic magnetic stirrer. Temperature was set to 37°C, and stirring speed to 180 rpm. At 4-h intervals, 100 μL samples were collected. After each collection, 10 μL internal standard and 190 μL methanol were added to the sample, which was then vortexed for 1 minute, followed by centrifuging at 16 000 g for 10 minutes. Finally, 10 μL supernatant was harvested for HPLC analysis. Each group’s experiment was performed in parallel 6 times.

In vivo protocol

SD rats (n = 12) were divided randomly into 2 groups for oral administration of SFE of ASR emulsion (500 mg/kg body weight) or Z-ligustilide emulsion (211 mg/kg body weight). Twelve hours before administration, rats were allowed to fast but drink ad libitum. Rats were etherized and blood samples were collected from the orbital sinus using a heparinized capillary tube with an inside diameter of 1.00 mm. Samples were collected in 1.5 mL heparinized Eppendorf tubes, and centrifuged at 3 000 g for 10 minutes. Plasma samples were harvested. Each plasma sample (100 μL) was mixed with 10 μL internal standard (α-naphthoflavone) and 190 μL methanol, vortexed, and centrifuged at 15 000 g for 5 minutes. The supernatant was filtered using a 0.45 μm syringe filter, and 150 μL of the filtrate was prepared for HPLC analysis. Each group experiment was performed in parallel 6 times.

Quantitative analysis

HPLC method: The HPLC system consisted of a solvent delivery unit (LC-20AT; Shimadzu (China) Co., Beijing), a UV/ VIS detector (SPD-20A; Shimadzu), an operating system software (LCsolution; Shimadzu), and an HPLC small molecule column (250 mm × 4.6 mm, 5 μm) coupled with a guard column (Grace Alltech Alltima, Beijing, China). The samples were eluted using a mobile phase at 20:80 water/ methanol, 0.7 mL/min. Z-ligustilide was monitored at a wavelength of 284 nm.
Calibration curves of Z-ligustilide: Methanol stock solution of Z-ligustilide (30 μg/mL) was diluted to the desired concentrations with methanol. A certain volume of the diluted solution was spiked into blank plasma from untreated SD rats to reach concentrations ranging from 0.25 to 15 mg/L for the in vivo study. The resultant samples were mixed thoroughly and treated and analyzed in the same manner as described under the in vivo protocol. Samples of each concentration were analyzed in triplicate. Calibration curves were derived by plotting the peak area ratios of Z-ligustilide to the internal standard as a function of concentration of Z-ligustilide.

Method validation: Analyses of bio-samples followed Food and Drug Administration (FDA) guidelines. Three concentrations of Z-ligustilide at high, medium, and low levels of the corresponding calibration curves were chosen to be determined for the intra-day and inter-day variability. The inter-day variability was determined on 3 separate days over 1 week. Peak area ratio for each concentration was measured, and concentration of Z-ligustilide was calculated from the corresponding calibration curve. Precision and accuracy of measures were evaluated by relative standard deviation and percentage difference between amounts spiked and determined (absolute recovery), respectively.

The concentration of a sample was at a single-to-noise ratio or higher than 5 was determined as the limit of quantitation (LOQ).

Identification of ligustilide in SFE and blood samples

Ligustilide levels in SFE of ASR and blood samples were determined using high-performance liquid chromatography mass spectroscopy (HPLC-MS) (LTQ Orbitrap XL, Thermo Scientific, Waltham, MA, USA).

Chromatography conditions: Mobile phase consisted of A (0.25% aqueous acetic acid, v/v) and B (methanol). Gradient elution was performed as follows: 30% B at 0–10 minutes, increased linearly to 60% at 20 minutes, to 90% at 50 minutes, and to 100% at 70 minutes. Phase A from 0% to 70% was at 80–90 minutes and maintained for 10 minutes. Flow rate was 0.7 mL/min.

Positive electrospray ionization MS was conducted under the following condition: 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, 5 500 V. Full scan mass spectrum was obtained over a range of m/z 50 through 600. Mass spectrum was recorded with Thermo Scientific QuanLab Forms 2.5 software.

Analysis of pharmacokinetic parameters

Pharmacokinetic parameters were calculated from the corresponding plasma concentration-time curves using noncompartmental analysis (WinNonlin 4.1, Pharsight, Mountain View, CA, USA). $C_{\text{max}}$, $T_{\text{max}}$, and $t_{1/2}$ were measured from the concentration-time profile. Other pharmacokinetic parameters, including $t_{1/2}$, $AUC_{0–\infty}$, $AUC_{0–t}$, $CL$, and $MRT_{0–\infty}$, were calculated using the following equations:

\[
AUC_{0–\infty} = AUC_{0–t} + \frac{C_{\text{last}}}{\lambda_2} \\
CL/F = \frac{Dose}{AUC_{0–\infty}} \\
MRT = AUMC_{0–\infty}/AUC_{0–\infty} \\
V_{ss}/F = \frac{Dose}{(\lambda_2 \times AUC_{0–\infty})}
\]

Where $\lambda_2$ is the terminal rate constant; $AUC_{0–t}$ is the area under the first moment curve from Time 0 to Time 8.

Statistical analysis

All data and results were expressed as mean ± SD. The t test was applied to compare results between two groups. Statistical significance was $P < 0.05$.

Results

Changes in levels of ligustilide in gastrointestinal contents

Levels of Z-ligustilide in its pure form as well as in SFE of ASR decreased in rat gastrointestinal contents (Fig. 2).

![Figure 2](image)

**Figure 2** Changes in levels of Z-ligustilide in gastrointestinal contents. (A) Changes in levels of Z-ligustilide in gastric contents; (B) Changes in levels of Z-ligustilide in intestinal contents. Data are mean ± SD, n = 6. *$P < 0.05$, **$P < 0.01$, comparison made between SFE group and ligustilide group.
Within 8 hours, 14.46% of Z-ligustilide in SFE of ASR and 19.9% of Z-ligustilide in its pure form were undetectable in gastric contents. In the intestinal contents, 9.85% of Z-ligustilide in SFE of ASR and 13.85% of Z-ligustilide in its pure form was undetectable. Significant difference in metabolism of Z-ligustilide between SFE of ASR and its pure form were not observed, but the decreasing rate of Z-ligustilide in its pure form was greater than in SFE of ASR. There was no significant difference in the amounts of degradation of Z-ligustilide between the gastric and intestinal contents.

Changes in level of ligustilide in liver tissue homogenates PMS

Within 4 hours, 70.45% and 80.02% of Z-ligustilide were metabolized in liver tissue PMS in SFE of ASR or in its pure form, respectively. Within 1.5 hours, 69.81% and 78.80% of Z-ligustilide (over 98% of metabolic amount), were metabolized in SFE of ASR or in its pure form, respectively (Fig. 3).

Method validation

HPLC was considered suitable for the quantification of Z-ligustilide in samples collected from in vivo studies (Fig. 4). Z-ligustilide was well separated from its metabolites. Over the concentration range tested, the calibration curves for plasma and in vitro samples showed good linearity ($r^2 > 0.998$). The overall intra-day and inter-day variations were less than 5% (0.42%–3.15%) (Table 1), and absolute recovery was higher than 79% (79.15%–84.16%) (Table 2). These results indicated good reproducibility and precision. LOQ of Z-ligustilide in rat plasma was 25 ng/L.

Figure 3  Changes in levels of Z-ligustilide in liver tissue homogenates PMS.

**$P < 0.01$, comparison made between SFE group and ligustilide group.

Within 8 hours, 14.46% of Z-ligustilide in SFE of ASR and 19.9% of Z-ligustilide in its pure form were undetectable in gastric contents. In the intestinal contents, 9.85% of Z-ligustilide in SFE of ASR and 13.85% of Z-ligustilide in its pure form was undetectable. Significant difference in metabolism of Z-ligustilide between SFE of ASR and its pure form

Figure 4  HPLC chromatograms of rat blank plasma (A) and spiked with Z-ligustilide and $\alpha$-naphthoflavone (B). 10 μL samples were ejected and eluted using a mobile phase contains A (80% methanol) and B (20% water) at 0.7 mL/min in HPLC. Z-ligustilide was monitored at a wavelength of 284 nm.
Identification of ligustilide in blood samples

Peak of Ligustilide was found using HPLC/MS analysis (Fig. 5). In Fig. 5, protonated molecular ion ([M+H]+) was at m/z 191 peak, deethylolation molecular ion was at m/z 161 peak, and depropylation molecular ion was at m/z 145 peak. Decarbonylation and break of lacton bond would produce the ion peak at m/z 134, m/z 121, m/z 106, and m/z 91 respectively. Peak at m/z 78 was from the molecular fragment of benzene. Chromatogram retention time of ligustilide was 39.56 minutes and NL was 3.92E7. Lysis started from the side chain, two collision cleavage pathways were butenyl losing and breaking of lactone bond with dehydration and decarbonylation (Fig. 6).

Pharmacokinetic study

Plasma concentration-time curves of Z-ligustilide after oral administration in its pure form or SFE of ASR were plotted (Fig. 7). Pharmacokinetic parameters were calculated and summarized (Table 3). The plasma concentrate–time profile and pharmacokinetic parameters could be obtained, only if the dose of SFE of ASR and Z-ligustilide administrated reached 500 mg/kg bodyweight and 211 mg/kg bodyweight. At doses of 100 mg/kg and 300 mg/kg, most response values of plasma samples were lower than LOQ, or even could not be detected. After oral administration, Z-ligustilide exhibited extensive distribution in the body (Vd, 339.53 ± 65.37 and 240.92 ± 76.12 L/kg) and rapid elimination (t1/2, 182.46 ± 79.78 and 199.82 ± 74.46) in its pure form or SFE of ASR, respectively. Z-ligustilide was absorbed rapidly (Tmax, 19.20 ± 15.26 and 17.43 ± 11.40 min) reaching a Cmax of 1.71 ± 0.27 and 1.32 ± 0.12. Within 3 hours, concentration of Z-ligustilide declined to 0.5 ng/L, a level which was maintained for the next 5 hours. Retention of Z-ligustilide (MRT, 258.64 ± 78.76 and 299.27 ± 114.6) reflects the long-lasting terminal phase. The value of total body clearance (CL) of Z-ligustilide in its pure form was significantly higher than that of in SFE of ASR (1.24 ± 0.21 versus 0.96 ± 0.16).

Discussion

A large number of drugs or active components have been studied for their chemo-preventive effect in experimental animals, and some of these drugs have even been applied

Table 1

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<td>RSD (%)</td>
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Clinically, chemopreventive agents can be divided into 2 types, synthetic agents and natural substances. Selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAID), and oral antidiabetic drug metformin are representative drugs of synthetic agents. Polyphenols from green tea, curcumin from ginger, sulforaphane from broccoli, resveratrol from grape and wine, and Z-ligustilide from angelica are also well studied as chemopreventive natural products. Long-term and constant usage has shown these vegetables, spices, and beverages are safe and reliable for human administration.

From the results of this study, the gastrointestinal surroundings, including pH, bioactive enzyme, and gastrointestinal microorganism, are not the notable factor influencing stability or metabolism of Z-ligustilide either in its pure form or in SFE of ASR. In spite of that, the 8 hours metabolism rate and decreasing rate of Z-ligustilide in its pure form were greater than in SFE of ASR. The interpretation for this phenomenon is that the co-existent components in SFE of ASR enhance the stability of Z-ligustilide.

<table>
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<th>Table 2</th>
<th>Recovery of ligustilide in blood samples (n = 6, %).</th>
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<tr>
<td>High</td>
<td>15</td>
</tr>
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</table>

Figure 5  HPLC chromatograms and Mass spectra of ligustilide in its pure form (A), blood sample (B), and SFE of ASR (C).
Based on the GC–MS analysis by our research group previously, isomerization of Z-ligustilide and clathration or covered up by co-existent components were inferred to be sound explanation for the improvement of Z-ligustilide stability in gastrointestinal contents.

Results from liver tissue homogenates PMS experiments indicated that Z-ligustilide regardless of in its pure form or in SFE of ASR was eliminated rapidly in the first 90 minutes. As for the ceasing reaction after 90 minutes, a rational explanation was that the lack of NADPH-regenerating system blocks the bio-redox cycle, so once the substrate exhausted, the reaction was forced into discontinuation. The overall trend of Z-ligustilide elimination was fully reflected within 1.5 hours liver tissue PMS metabolic experiments, and the conclusion of liver being the main metabolic organ for Z-ligustilide can be validated.

Pharmacokinetic study indicated that ligustilide was absorbed apace (T\text{max}; 17.43 ± 11.40 min) and eliminated

![Figure 6](image6.png)  
Figure 6 A proposed lysis pathway of Ligustilide in the rat. Lysis was started from the side chain, two collision cleavage pathway were butenyl losing and breaking of lactone bone with dehydroation and decarbonylation.

![Figure 7](image7.png)  
Figure 7 Mean plasma concentration-time profiles of Z-ligustilide in rat after oral administration.

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![Figure 8](image8.png)  
Figure 8 Main metabolites of ligustilide in rat. Gly: glycine; Cys: cysteine; Glu: glutamate.

**Table 3** Pharmacokinetic parameters of Z-ligustilide after oral administration (n = 6).

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
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<th>Z-Ligustilide (0.211 µg/mL)</th>
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<tr>
<td>T\text{max} (min)</td>
<td>19.20 ± 15.26</td>
<td>17.43 ± 11.40</td>
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<tr>
<td>C\text{max} (mg/L)</td>
<td>1.71 ± 0.27*</td>
<td>1.32 ± 0.12</td>
</tr>
<tr>
<td>t\text{/2} (min)</td>
<td>182.46 ± 79.78</td>
<td>199.82 ± 74.46</td>
</tr>
<tr>
<td>V\text{b}/F (L/kg)</td>
<td>339.53 ± 65.37*</td>
<td>240.92 ± 76.12</td>
</tr>
<tr>
<td>AUC\text{0–inf} (mg/L)</td>
<td>293.43 ± 44.26*</td>
<td>228.28 ± 42.00</td>
</tr>
<tr>
<td>AUC\text{0–inf} (mg/L)</td>
<td>243.37 ± 16.84*</td>
<td>176.69 ± 12.59</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>0.96 ± 0.16*</td>
<td>1.24 ± 0.21</td>
</tr>
<tr>
<td>MRT\text{0–inf} (min)</td>
<td>258.64 ± 78.76*</td>
<td>299.27 ± 114.36</td>
</tr>
</tbody>
</table>

Note: * P < 0.05 comparison made between SFE group and ligustilide group.
rapidly ($t_{1/2} = 199.82 \pm 74.46$ min). The clearance of Z-ligustilide after administration in its pure form was significantly lower than that dose in SFE of ASR (CL, 0.96 ± 0.16 mL·min/kg versus 1.24 ± 0.21 mL·min/kg ($P < 0.05$); AUC, 243.37 ± 16.84 versus 176.69 ± 12.59 mg·min/L). Combined with the results of metabolism study in liver tissue homogenates PMS and in gastrointestinal contents, the conclusion could be obtained that the extensive first pass metabolism in liver is one of the reasons of low oral bioavailability of Z-ligustilide.

From the results of this study, the stability of Z-ligustilide was improved in SFE of ASR in gastrointestinal contents. Metabolic rate of Z-ligustilide in SFE of ASR after oral administration showed greater AUC on concentration-time profiles than in its pure form. The reason may be that the co-existing components in SFE improved the stability of Z-ligustilide and moderated the pharmacokinetic characteristics.

Ligustilide is metabolized mostly through aromatization, hydration, oxidation, and glutathione conjugation (Fig. 8). Further research is needed to explore the specific interaction between Z-ligustilide and the co-existing components in SFE of ASR. Aspects that we intend to look into include the conclusion effect of co-existing components to Z-ligustilide and isomerization equilibrium of Z-ligustilide and its isomer such as butylidenephthalide. In addition, though the stability of Z-ligustilide was improved, its serum concentration during a certain time period was still not maintained. Therefore, further pharmacetic processing is also warranted in the development of SFE of ASR for chemoprevention of colorectal cancer. Using a new delivery system to change the route of administration and developing sustained and controlled release preparations are possible solutions to avoid the first-pass effect and improve bioavailability.

Acknowledgments

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References