ORIGINAL RESEARCH

High hepatic exposure to ferulic acid, ligustilide, and ligustrazine in Chuanxiong Rhizome is associated with transporter-mediated active uptake

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Abstract

Objectives

To investigate the in vivo tissue distribution and hepatic exposure mechanisms of ferulic acid, ligustrazine, and (Z)-ligustilide, the main active constituents of Chuanxiong Rhizome, and to elucidate the transport mechanisms involved in their hepatic uptake.

Method

Chuanxiong Rhizome was extracted using supercritical fluid extraction, and the concentrations of ferulic acid, ligustrazine, and (Z)-ligustilide were quantified by high-performance liquid chromatography and identified by secondary mass spectrometry. Male rats were administered the extract intragastrically at a dose of 1.5 g/kg. Blood, liver, spleen, lungs, and kidneys were collected at designated time points post-administration to determine tissue concentrations of the compounds. Hepatic uptake studies were performed using isolated normal rat hepatocytes obtained via the two-step collagenase perfusion method. The effects of temperature, metabolic inhibitors, and specific transporter inhibitors on hepatic uptake were evaluated to clarify transport mechanisms.

Results

All three compounds were rapidly distributed after oral administration, with significantly higher exposure observed in the liver compared with other tissues. Hepatocyte uptake was temperature-dependent and markedly reduced by metabolic and transporter inhibitors, indicating an active transport process. Ferulic acid uptake was primarily mediated by organic anion transporters, while ligustrazine uptake involved organic cation transporters. (Z)-Ligustilide showed notable hepatic accumulation, suggesting transporter-associated uptake, although its specific transport mechanism was less clearly defined.

Conclusion

Ferulic acid, ligustrazine, and (Z)-ligustilide from Chuanxiong Rhizome exhibit pronounced liver exposure following oral administration. This preferential hepatic distribution is largely attributed to active transport mechanisms, with organic anion and cation transporters playing key roles in the hepatic uptake of ferulic acid and ligustrazine, respectively. These findings provide mechanistic insight into the liver-targeting characteristics and pharmacological actions of Chuanxiong Rhizome.

Keywords: Chuanxiong Rhizome, Ferulic acid, ligustrazine, (Z)-ligustilide, hepatic exposure.

Introduction

Chuanxiong Rhizome, a widely-utilized traditional Chinese herbal medicine sourced from the dried rhizome of Ligusticum chuanxiong Hort[1]. It has long been a common ingredient in various Chinese medicine preparations. For thousands of years, Chuanxiong Rhizome was initially acknowledged as a safe and effective medicinal material for treating cardiovascular and cerebrovascular diseases[2]. In recent years, its therapeutic scope has expanded. However, rather than simply listing its new-found effects, it's crucial to note that these discoveries have set the stage for our current research. Chuanxiong Rhizome has been shown to facilitate pain relief[3], treat endocrine diseases[4], and gynecological diseases[2]. This broadening of its therapeutic applications has sparked further interest in understanding the underlying mechanisms, which is precisely what our study aims to address.

Extensive research efforts have been dedicated to isolating and identifying the chemical components within Chuanxiong Rhizome. As a result, more than two hundred monomeric components have been identified, including alkaloids, volatile oils, phenolic compounds, and lactones[5]. Among these, ferulic acid, ligustrazine, and (Z)-ligustilide stand out as the main active ingredients that contribute to the action mechanism of Chuanxiong Rhizome in clinical practice. Ferulic acid, a derivative of cinnamic acid with the chemical structure of 3-methoxy-4-hydroxycinnamic acid, has a range of physiological activities[6]. But instead of just listing these effects, we should emphasize their relevance to our research focus. Its antioxidant properties, anti-platelet aggregation, anti - thrombosis, myocardial protection, and anti-lipid peroxidation[7] are all factors that may influence its distribution and biological behavior in the body, which is a key aspect of our study.

Ligustrazine (2,3,5,6-tetramethylpyrazine) is the most important alkaloid in Chuanxiong Rhizome. It can rapidly and effectively alleviate ischemic cardiovascular and cerebrovascular conditions[8]. Its various clinical effects, such as vasodilation, inhibition of platelet aggregation, prevention of thrombosis, and improvement of cerebral ischemia,

are not just isolated phenomena. These effects are closely related to how ligustrazine is distributed and functions within the body, which is a central concern of our research[9]. Therefore, it is mainly used for the treatment of ischemic cerebrovascular diseases in clinical practice.

(Z)-ligustilide is the highest - content benzoquinone derivative in Chuanxiong Rhizome, accounting for about 1.6% of the crude medicinal material[10]. Similar to the other two active ingredients, its properties and potential interactions within the body are of great interest to our study.

The liver is one of the main organs responsible for drug metabolism. Drug exposure in the liver significantly affects the efficacy, toxicity, and interaction of other drugs[11]. To date, due to the uncertainty and complexity of active ingredients in herbal products, as well as the insufficient understanding of the pharmacokinetics of ferulic acid, ligustrazine, and (Z)-ligustilide, the hepatic exposure and mechanism of active ingredients in Chuanxiong Rhizome remain unclear.

This study aimed to investigate the in vivo tissue distribution and hepatic exposure mechanisms of ferulic acid, ligustrazine, and (Z)-ligustilide, the main active ingredients of Chuanxiong Rhizome.

Materials and Methods

Materials

Chuanxiong Rhizome, originating from Aoping Dujiangyan, was purchased from Guangzhou Sanxian Hecheng Biotechnology Co., LTD and identified by the Beijing Institute of Radiation Medicine. The reference standards of ferulic acid, ligustrazine, and (Z)-ligustilide were supplied by the Yuanye Biotechnology Co., LTD (Shanghai, China). The purity of each reference standard was more than 98%.

Preparation of chuanxiong rhizome extract

Chuanxiong Rhizome was initially chopped into small pieces and ground into raw powder, which was then filtered through a 30-mesh sieve. The powder was immersed in water for 5 h and subsequently mixed twice with a 75% ethanol aqueous solution at a ratio of 1:10 (w/v). Next, the powder underwent supercritical fluid CO_2 extraction using a HA121-50-01 supercritical fluid CO_2 system (Anhui, Nantong, China). The extraction was carried out under a pressure of 45 MPa and a temperature of 55°C for 3.5 h. After extraction, the extraction solutions were filtered to remove particulates and concentrated at 45°C under reduced pressure. Then, the extract was diluted with a 0.5% (v/v) CMC-Na solution to achieve a final concentration of 1.5 g/mL of Chuanxiong Rhizome extract.

Quantitatively and qualitatively measurement of extract

To determine the extraction yield or efficiency of the key compounds (ferulic acid, ligustrazine, and (Z)-ligustilide) in the supercritical CO_2 extraction, we first established standard curves for each compound using HPLC. The standard curves were constructed by plotting the peak areas of known concentrations of the standards against their corresponding concentrations.

We accurately weighed a certain amount of the initial Chuanxiong Rhizome powder (denoted as *minitial−powder,* in grams) before the supercritical CO_2 extraction. After the extraction and subsequent concentration steps, we obtained the concentrated extract. We then took an aliquot of the concentrated extract and diluted it appropriately to a volume *Vdiluted* (in mL) for HPLC analysis. The contents of ferulic acid, ligustrazine, and (Z)-ligustilide in the extract were quantitatively measured by HPLC equipped with a UV detector and a SHIMADZU Shim-pack GIST C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was an acetonitrile - 0.5% glacial acetic acid aqueous solution, and a gradient elution procedure was applied as shown in Table 1. The Chuanxiong Rhizome extract was thoroughly dissolved in the mobile phase with sonication for 20 min. A 10 μL sample was injected into the HPLC system, and each test solution was replicated and determined three times. The column temperature was set at 30°C, and the HPLC chromatograph was monitored at 285 nm.

The contents of ferulic acid, ligustrazine, and (Z)-ligustilide in the extract were quantitatively measured by HPLC equipped with a UV detector and SHIMADZU Shim-pack GIST C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was acetonitrile-0.5% glacial acetic acid aqueous solution, gradient elution procedure was applied according to Table 1, the Chuanxiong Rhizome extract was thoroughly dissolved in the mobile phase with sonication for 20 min. 10 μL of sample was injected into the HPLC system, each test solution was replicated and determined three times; The column temperature was set at 30°C; the HPLC chromatograph was monitored at 285 nm.

Based on the peak areas obtained from the HPLC analysis of the diluted extract samples and the standard curves, we calculated the concentrations of ferulic acid (Cferulic−diluted, in mg/mL), ligustrazine (*Cligustrazine−diluted*, in mg/mL), and (Z)-ligustilide ($C_{(Z)}$*−ligustilide*−diluted, in mg/mL) in the diluted extract. Then, we calculated the total amounts of these compounds in the concentrated extract: The total amount of ferulic acid in the concentrated extract, mferulic−concentrated = *Cferulic−diluted* × Vdiluted (in mg). The total amount of ligustrazine in the concentrated extract, mligustrazine−concentrated = *Cligustrazine−diluted* × Vdiluted (in mg). The total amount of (Z)-ligustilide in the concentrated extract, *m(Z)−ligustilide−concentrated* = C(Z)−ligustilide−diluted × Vdiluted (in mg).

We also determined the initial amounts of these compounds in the Chuanxiong Rhizome powder through a separate pre-experiment using the same HPLC method. Let the initial amounts of ferulic acid, ligustrazine, and (Z)-ligustilide in the initial powder be mferulic−initial, mligustrazine−initial, and m(Z)−ligustilide−initial (in mg) respectively. The extraction yield of each compound was calculated using the following formula: Extraction yield (%) = (*mcompound−initial*/*mcompound−concentrated*)×100%. After performing the experiments and calculations, we found that the extraction yield of ferulic acid was 86.7%, the extraction yield of ligustrazine was 82.5%, and the extraction yield of (Z)-ligustilide was 75.6%.

The peaks of ferulic acid, ligustrazine and (Z)-ligustilide were isolated and recycled to further MS analysis. Samples were electrospray ionized with the following optimized conditions: Electrospray ionization voltage was 5500 V; Temperature was 580°C; Source gas-1 (N2) pressure was 50 psi; Gas 2 (N2) pressure was 55 psi; Curtain gas pressure was 15 psi; The scanning method is multiple reaction monitoring with medium collision gas (N2) pressure; The dissociation voltage was 48 V; The collision energy was 20 eV; The selected transitions for (Z)-ligustilide was m/z 190.07 →

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Table 1. Gradient elution procedure of HPLC

Time (min)	Velocity (mL/min)	Acetonitrile (%)	0.5% glacial acetic acid (%)
0.00	1	10	90
15.00	1	10	90
30.00	1	20	80
35.00	1	30	70
40.00	1	55	45
45.00	1	55	45
55.00	1	95	5
60.00	1	95	5
65.00	1	10	90
70.00	1	10	90

Table 2. Gradient elution procedure of HPLC

Time (min)	Velocity (mL/min)	Acetonitrile (%)	Water (%)
0.00	1.1	20	80
0.10	1.1	20	80
0.80	1.1	97	3
2.50	1.1	97	3
2.51	1.1	20	80
4.50	1.1	20	80

Table 3. Mass spectrum characteristics of the three compounds

Item	Ferulic acid	(Z)-ligustilide	Ligustrazine
Retention time (min)	24.6±0.5	43.9±0.6	30.4±0.4
Parent ion peak	365±5	190.0±3	136.2±2

Table 4. LC/MS validation results

Item	Ferulic acid	(Z)-ligustilide	Ligustrazine
Linear range	31.8-2544 ng/mL	5.35-2500 ng/mL	6.42-2153 ng/mL
Lower limit of quantification	31.8 ng/mL	5.35 ng/mL	6.42 ng/mL
Intra-batch precision values (RSD%)	8.3±1.2%	7.4±1.0%	9.3±1.5%
Inter-batch precision values (RSD%)	5.6±0.8%	4.3±0.6%	6.0±0.9%
Accuracy values (RE%) for low, medium, and high levels of quality control samples	11.3%-12.0%	9.0%-10.4%	10.3%-12.1%
Mean extraction recoveries	89±5%	91±4%	92±3%

Table 5. AUC0-∞ and Cmax of ferulic acid, ligustrazine, and (Z)-ligustilide in plasma and tissues

	Cmax (μg/g)			AUC0-∞		
	Ferulic acid	Ligustrazine	(Z)-ligustilide	Ferulic acid	Ligustrazine	(Z)-ligustilide
Plasma	0.31±0.05	0.57±0.08	0.89±0.10	462±45	875±86	1642±165
Liver	1.64±0.22	2.15±0.30	4.62±0.53	2642±265	4011±400	8742±852
Kidney	1.06±0.15	1.18±0.17	3.11±0.35	1548±156	2738±274	6324±641
Lung	0.48±0.07	0.50±0.07	2.05±0.23	1379±138	1246±126	5320±536

161.03, ligustrazine was m/z 190.08 → 161.04, ferulic acid was m/z 291.07 → 249.03.

Rat treatment

Male rats (Sprague-Dawley, 180-220 g, Jackson Laboratory) were obtained from the Animal Central of the Institute (Beijing, China). All the animals were bred with a standard diet in an SPF environment. Rats that experienced absolute fasting for 12 h were randomly divided into four groups (5 rats per group). All rats were intragastric administrated one dose of 1.5 g/kg Chuanxiong Rhizome extract (equivalent to 16.74 g/kg of raw material, 2.92 mg/kg ferulic acid, 0.23 mg/kg of ligustrazine and 12.86 mg/kg of (Z)-ligustilide). Blood, liver, kidney, spleen and lung tissue samples were collected from rats at 15 min, 1 h, 4 h, and 8 h after dosing. One extra rat was sacrificed to provide blank control samples. The liver tissues were obtained by surgery and immediately frozen at −80°C until subsequent extraction. All animal procedures were approved by the Ethics Committee of

Guangdong Second Provincial General Hospital, and the approval number was GD2024-0018[L].

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Table 6. $K_{p,Cmax}$ and $K_{p,AUC}$ of ferulic acid, ligustrazine, and (Z)-ligustilide

	$K_{p,Cmax}$			$K_{p,AUC}$		
	Ferulic acid	Ligustrazine	(Z)-ligustilide	Ferulic acid	Ligustrazine	(Z)-ligustilide
Liver	5.3±0.7	3.8±0.5	5.2±0.5	5.7±0.6	4.6±0.5	5.3±0.6
Kidney	3.4±0.5	2.1±0.3	3.5±0.4	3.3±0.4	3.1±0.6	3.9±0.4
Lung	1.5±0.2	0.9±0.1	2.3±0.3	3.0±0.3	1.4±0.2	3.2±0.3
Spleen	1.9±0.3	0.4±0.1	1.3±0.3	0.2±0.03	0.7±0.08	1.1±0.2

Treatment of plasma and tissue sample

For plasma sample treatment, 5 replicates were involved in total. Rat plasma was mixed with 20 μL of internal standard solution and 660 μL of acetonitrile. The solution was vortex oscillated for 5 min and centrifuged at 4°C and 15000 r/min for 10 min. 750 μL of supernatant was extracted with 200 μL of 50% acetonitrile after being blown to dry with nitrogen. Afterward, the supernatant was centrifuged at 4°C and 15000 r/min for 15 min and collected to serve as an analytical sample.

For each tissue type (liver, kidney, lung, and spleen), there were 20 samples in total. The liver, kidney, lung, and spleen tissue of each rat was immediately cut off washed with normal saline and weighed. Mix the tissue with 3-fold the amount of normal saline containing 10% ammonia. The tissue was homogenized at 20000 r/min in an ice bath. 300 μL homogenate was mixed with the 30 μL of internal standard solution, and 1470 μL of acetonitrile.

The solution was vortex-mixed for 5 min and centrifuged at 4°C and 13000 r/min for 15 min. After being blown to dry with nitrogen, the supernatant was extracted with 1.8 mL of ethyl acetate methanol (100:1), then centrifuged at 4°C and 13000 r/min for 15 min. The supernatant was blown to dry with nitrogen and then mixed with 250 μL of 50% acetonitrile as an analytical sample.

Hepatic uptake of the ferulic acid, ligustrazine, and (Z)-ligustilide was performed in normal rat hepatocytes by Seglen two-step collagenase perfusion method 12. Blood cells in rat liver tissue were removed with perfusion solution A (20 mmol/L Hepes, 200 U/mL penicillin/streptomycin, 1.25 mmol/L EDTA, dissolved in D'Hanks solution). Tissue was digested with perfusion solution B (1 mg/mL collagenase IV, 5 mmol/L calcium chloride, dissolved in Hank's solution) at 37°C. Tissue was filtered, and centrifuged, and collected the cell precipitation until the supernatant gradually became clear. Subsequently, the liver parenchymal cells were resuspended with Williams' E complete culture medium containing 10% fetal bovine serum, 1 × penicillin/streptomycin, and 1 × Glutamax). The hepatocytes were cultivated in fully humidified air with 5% CO_2 at 37°C. Trypan blue exclusion tests were performed to screen hepatocytes with a cell viability of more than 85%.

Quantification analysis for ferulic acid, ligustrazine, and (Z)-ligustilide

Ferulic acid, ligustrazine and (Z)-ligustilide in plasma, liver, kidney, lung and spleen samples were quantitative measured using 3200 QTrap-Tandem LC/MS system (Applied Biosystems, USA) equipped with electrospray ionization Source (ESI) and DB-5MS column (30 m × 0.25 mm × 0.25 μm); The mobile phase was 50% acetonitrile-water solution, gradient elution procedure was applied according to Table 2 after 0.5 min of re-equilibration. The injection volume was 10 μL; the Detection wavelength was 285 nm; The column temperature was 30°C. The analytical method was validated for the linear, lower limit of quantification, intra-batch, and inter-batch precision values, accuracy and mean extraction recoveries. The experiment was repeated 3 times.

Hepatic uptake in vitro and kinetic measurement

A concentration of 1×106 cells/mL of rat primary hepatocytes was added to the DMEM-F12 medium introduced into 2 mmol/L 1-amino benzotriazole, then incubated for 15 minutes to inhibit CYP enzyme activity. A solution containing ferulic acid, ligustrazine, and (Z)-ligustilide (10 μM) was added to cells and incubated at either 4°C or 37°C for 1, 3, 6, 9, or 12 min to observe hepatocyte uptake. Besides, solutions containing different concentrations of ferulic acid, ligustrazine, and (Z)-ligustilide at 0 μM, 10 μM, 20 μM, 40 μM, and 80 μM were added separately to cells and incubated at 37°C for 12 min to observe hepatocyte uptake on concentration manner. To collect a sample for uptake kinetic analysis, the cells incubated at 37°C for 1 h were separated and sampled.

A total of 100 μL samples were placed in an Easy Cut microcentrifuge tube containing 100 μL 0.1% acetic acid on 10% methanol. The samples were immediately centrifuged at high speed for 1 minute to collect the cell precipitate, The cells were transferred into a container containing 300 μL acetonitrile. GC-MS/MS was performed to analyze the concentration of ferulic acid, ligustrazine, and (Z) - ligustilide in liver cells.

The equation used to calculate the initial rate of hepatic uptake (V) was as follows:

$$V = \frac{V_{max} \times S}{K_m + S} + P_{diff} \times S$$

Vmax is the maximum rate of hepatic uptake. It is the theoretical upper limit of the rate at which the liver can take up the active ingredients (ferulic acid, ligustrazine, or (Z)-ligustilide) when the substrate concentration is extremely high and all the available transporters are saturated. In our experimental context, it indicates the highest possible rate at which the rat primary hepatocytes can internalize these substances under optimal conditions.

S is the initial concentration of ferulic acid, ligustrazine, or (Z)-ligustilide in the incubation medium. It is a variable in the equation and can take on different values depending on the experimental setup. In our study, we tested different initial concentrations (0 μM, 10 μM, 20 μM, 40 μM, and 80 μM) to observe how the uptake rate changes with varying substrate levels.

Km is the substrate concentration at which the hepatic uptake rate (V) is equal to 50% of the *Vmax*. It is a measure of the affinity of the transporters in the hepatocytes for the active ingredients. A lower Km value indicates a higher

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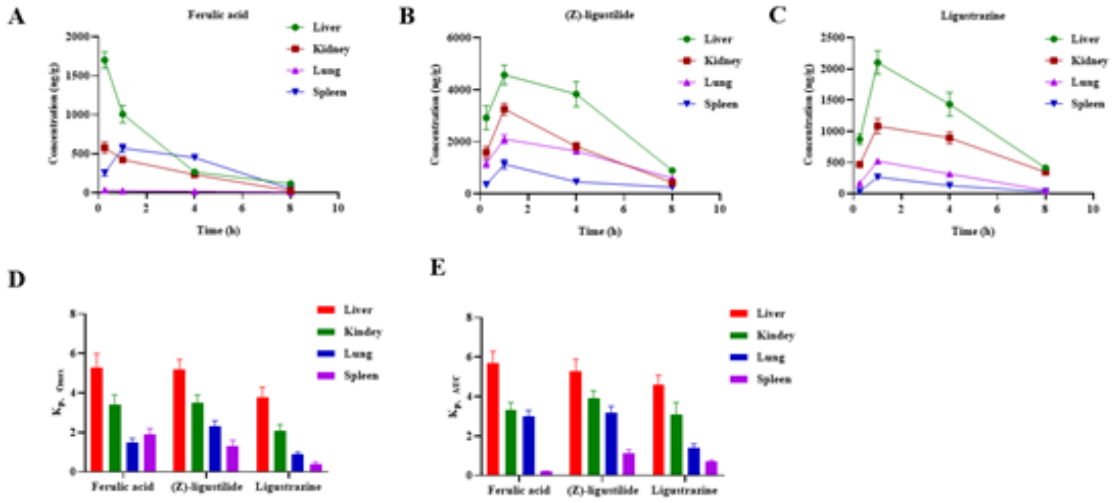

Fig. 1 Tissue distribution profiles of compounds in chuanxiong rhizome; (A) The uptake of ferulic acid after oral administration in different organs. (B) The uptake of ligustrazine after oral administration in different organs. (C) The uptake of (Z)-ligustilide after oral administration in different organs. (D) Kp,Cmax of ferulic acid, ligustrazine, or (Z)-ligustilide in different organs. (E) Kp,AUC of ferulic acid, ligustrazine, or (Z)-ligustilide in different organs

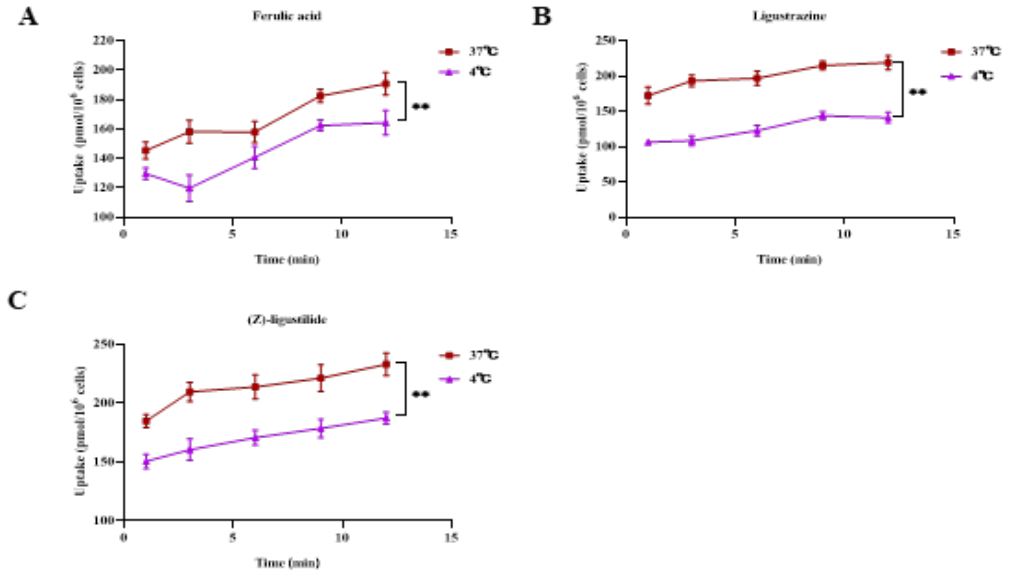

Fig. 2 The influence of different culture temperatures on hepatic uptake; (A) The influence of different culture temperatures on hepatocellular uptake of ferulic acid. (B) The influence of different culture temperatures on hepatocellular uptake of ligustrazine. (C) The influence of different culture temperatures on hepatocellular uptake of (Z)-ligustilide.

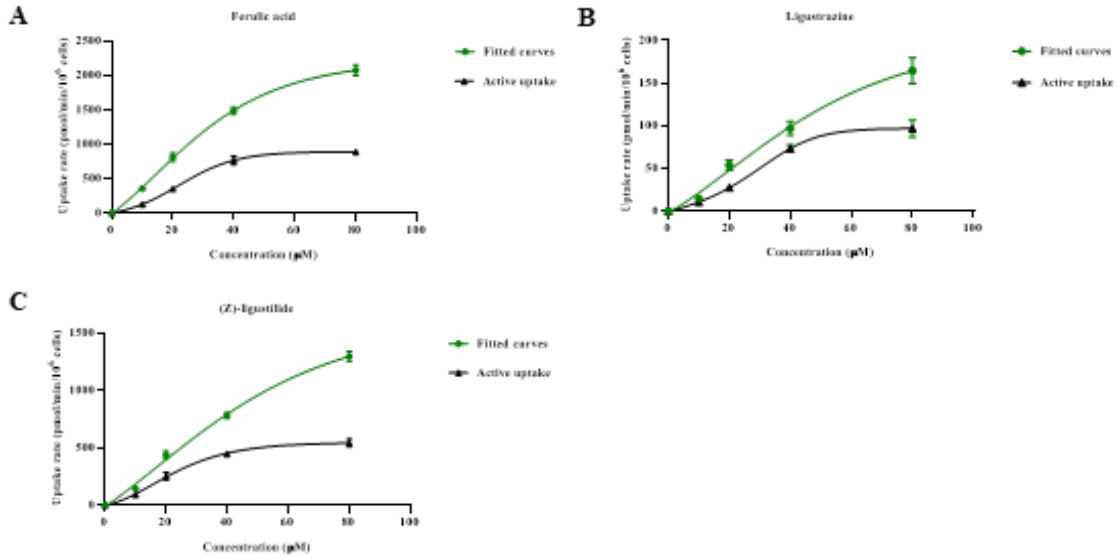

Fig. 3 The influence of different concentrations on hepatic uptake; (A) The influence of different concentrations on hepatocellular uptake of ferulic acid. (B) The influence of different concentrations on hepatocellular uptake of ligustrazine. (C) The influence of different concentrations on hepatocellular uptake of (Z)-ligustilide.

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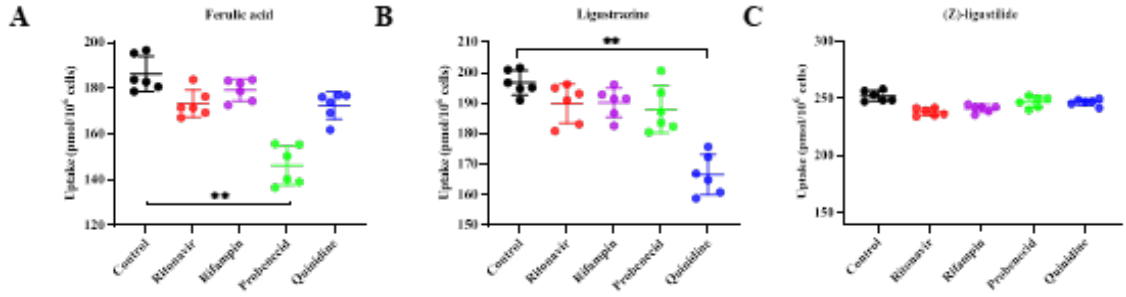

Fig. 4 The hepatic uptake after co-incubation with a series of transporter inhibitors; (A) The hepatic uptake of ferulic acid after co-incubation with a series of transporter inhibitors. (B) The hepatic uptake of ligustrazine after co-incubation with a series of transporter inhibitors. (C) The hepatic uptake of (Z)-ligustilide after co-incubation with a series of transporter inhibitors. **: P<0.01.

affinity of the transporters for the substrate, meaning that the transporters can achieve half of their maximum uptake rate at a lower substrate concentration.

P_{diff} represents the non-saturable clearance via passive diffusion. Passive diffusion is a process where substances move across the cell membrane from an area of high concentration to an area of low concentration without the need for energy or specific transporters. The P_{diff} value quantifies the rate at which the active ingredients enter the hepatocytes through this passive mechanism.

The modeling of hepatocyte uptake was estimated using the above kinetic parameters to distinguish between transporter-mediated uptake and passive diffusion. To estimate these parameters (V_{max}, *Km, and* P_{diff}), we employed MULTI, a non-linear least-squares analysis software. This method is based on minimizing the sum of the squared differences between the observed experimental data and the values predicted by the kinetic model.

In our experiment, we collected data on the hepatic uptake of ferulic acid, ligustrazine, and (Z)-ligustilide at different initial substrate concentrations (S) and different time points.

We then input these data into the MULTI software along with the kinetic equation. The software iteratively adjusted the values of V_{max}, K_m, and P_{diff} to find the set of values that best fit the experimental data. This process is known as curve-fitting, and it allows us to obtain accurate estimates of the kinetic parameters that describe the hepatic uptake process.

The experiment was repeated 3 times to ensure the reliability and reproducibility of the results. The data from these repeated experiments were used collectively for the parameter estimation process, which helped to reduce experimental errors and increase the accuracy of the estimated parameters[13].

Transepithelial transport study

To analyze the mechanism of liver uptake of active ingredients in Chuanxiong Rhizome, normal rat hepatocytes were separately treated with a series of transporter inhibitors to inhibit the uptake transporters activity in *vitro*: organic anion transporting polypeptide inhibitors Ritonavir and Rifampin, organic cation transporter inhibitor Quinidine, organic anion transporter inhibitor Probenecid.

A concentration of 1×106 cells/mL of rat hepatocytes was added to the DMEM-F12 medium introduced into 2 mmol/L 1-amino benzotriazole, and then incubated for 5 minutes to inhibit CYP enzyme activity. Ritonavir (10 μM), rifampin (10 μM), probenecid (100 μM), or quinidine (100 μM) were separately added to cells and incubated at 37°C for 15 min as reported previously[14-16]. A solution containing ferulic acid, ligustrazine, or (Z)-ligustilide (10 μM) was added to cells and incubated at 37°C for 5 min to observe hepatocyte uptake.

To ensure the reliability and reproducibility of our results, the transporter inhibition experiments were repeated three times independently. Each independent experiment was carried out with a fresh batch of rat hepatocytes prepared under identical conditions to minimize batch-to-batch variability. In each repetition, the same experimental procedures, including cell preparation, inhibitor treatment, and incubation with the active ingredients, were strictly followed.

For reproducibility confirmation, we analyzed the data from the three independent experiments. The results showed consistent trends in the uptake of ferulic acid, ligustrazine, and (Z)-ligustilide under different transporter inhibitor treatments across the three repetitions. The standard deviation and coefficient of variation were calculated for the measured concentrations of these active ingredients in liver cells in each group. The relatively low values of these statistical parameters indicated that the experimental results were reproducible and reliable.

Finally, samples were collected for GC-MS/MS analysis to determine the concentration of ferulic acid, ligustrazine, and (Z)-ligustilide in liver cells.

Statistical analysis

All the experiments were repeated at least three times. Compound uptake was analyzed by ANOVA with repeated methods to verify the normal distribution of the data. Data are all presented as mean ± S. GraphPad Prism 8.0 software was applied to analyze the results. Comparisons between two groups were carried out by the unpair t-test, and comparisons between multiple groups were carried out by one-way ANOVA. A two-sided p-value of less than 0.01 was considered statistically significant.

Results

Qualitative and Quantitative Analysis of Ingredients of Extract

HPLC was successfully applied for the fingerprint of extract and determination of concentrations of ferulic acid, ligustrazine, and (Z)-ligustilide in Chuanxiong Rhizome extract. The concentrations of ferulic acid, ligustrazine, and (Z)-ligustilide were respectively found to be 1.95, 0.15, and 8.57 mg/g extract. Ferulic acid, ligustrazine, and (Z)-ligustilide were separated and identified by secondary MS to verify the right structure, mass spectrum characteristics of ferulic acid were described as Table 3. The mass spectrum characteristics were consistent with the literature[17]. We confirmed ferulic acid, ligustrazine, and (Z)-ligustilide

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were successfully extracted from Chuanxiong Rhizome by supercritical fluid extraction.

Validation of the HPLC method

HPLC method was used to determine the concentrations of ferulic acid, ligustrazine, and (Z)-ligustilide in rats after oral administration. This method was validated to verify its applicability, the results are listed in Table 4.

Tissue Distribution Profiles of Compounds in Chuanxiong Rhizome

After rats received a dose of 1.5 g/kg Chuanxiong Rhizome extract, the concentration results of ferulic acid, ligustrazine, and (Z)-ligustilide revealed the fast absorption rate in liver, spleen, lung, and kidney from 25 min to 8 h after dosing. The three compounds had differentiated absorption profiles among tissues according to exposure parameter AUC0-∞ and listed Cmax in Table 5, liver was the main exposure organ of ferulic acid, ligustrazine, and (Z)-ligustilide in vivo. The exposure amount from most to least was (Z)-ligustilide, ligustrazine, and ferulic acid. The concentrations of ferulic acid occupy a height position at 25 min in most of the tissues (Fig. 1A), while ligustrazine and (Z)-ligustilide had the highest exposure at 1h among all tissues (Fig. 1B). Among the tissues, there was the most exposure of ferulic acid in liver at 25 min, 1 h and 8 h ($P<0.05$). Ligustrazine, and (Z)-ligustilide showed a similar trend that liver exposure was highest among the four tissues at 25 min and 1h (Fig. 1B & 1C).

Cmax and AUC0-∞ above were used to calculate the plasma partition coefficients (Kp) of ferulic acid, ligustrazine, and (Z)-ligustilide by regression equations to predict drug concentration ratios in important tissue-to-plasma. The liver Kp, Cmax of ferulic acid, ligustrazine, and (Z)-ligustilide were all significantly larger than those of the kidney, lung, and spleen (Fig. 1D), while the liver Kp, AUC of ferulic acid, ligustrazine, and (Z)-ligustilide were also higher compared to the Kp, AUC of other tissues (Fig. 1E). The results illustrated the high hepatic exposure at liver of ferulic acid, ligustrazine, and (Z)-ligustilide.

(A) The uptake of ferulic acid after oral administration in different organs. (B) The uptake of ligustrazine after oral administration in different organs. (C) The uptake of (Z)-ligustilide after oral administration in different organs. (D) Kp,Cmax of ferulic acid, ligustrazine, or (Z)-ligustilide in different organs. (E) Kp,AUC of ferulic acid, ligustrazine, or (Z)-ligustilide in different organs.

Hepatic Uptake Results

To study the mechanism for the high hepatic uptake of Chuanxiong Rhizome extract, rat hepatocytes incubated with the standard substance of ferulic acid, ligustrazine, and (Z)-ligustilide were served to measure the drug uptake profile. The hepatic uptake over different incubation temperatures showed that the uptake of ferulic acid, ligustrazine, and (Z)-ligustilide at 37°C in 1 h, 3 h, 6 h, 9 h, and 12 h were all significantly higher compared to those at 4°C ($P < 0.05$) (Fig. 2A-2C). Previous studies reported that the effect on active uptake into and out of cells mediated by transporters positively depended on temperature[18]. Our data demonstrated that the uptake of ferulic acid, ligustrazine, and (Z)-ligustilide may be an active process mediated by transporters.

Next, the effect of substrate concentration on uptake kinetics was characterized by a similar experiment design. The results revealed that the hepatic uptake rate of ferulic acid, ligustrazine, and (Z)-ligustilide were all characterized by concentration-dependent raising over a range from 10 μM to 80 μM, the higher substrate concentration, the faster uptake rate could bear (Fig. 3A-3C).

Fig. 2 The influence of different culture temperatures on hepatic uptake

(A) The influence of different culture temperatures on hepatocellular uptake of ferulic acid. (B) The influence of different culture temperatures on hepatocellular uptake of ligustrazine. (C) The influence of different culture temperatures on hepatocellular uptake of (Z)-ligustilide.

Fig. 3 The influence of different concentrations on hepatic uptake

(A) The influence of different concentrations on hepatocellular uptake of ferulic acid. (B) The influence of different concentrations on hepatocellular uptake of ligustrazine. (C) The influence of different concentrations on hepatocellular uptake of (Z)-ligustilide.

The Effect of Transporter Inhibitors on Hepatic Uptake Change

A panel of known transporter inhibitors was co-incubated with hepatocytes to study the influence on the hepatic absorption of ferulic acid, ligustrazine, and (Z)-ligustilide to explore if drug transporters have any effect on the hepatic uptake of the three compounds. According to the results, the cellular content of ferulic acid was all significantly decreased from 183.4±15.2 pmol/10^6 cells to 145.4±12.3 pmol/106 cells when co-incubated with Probenecid, a reduction of approximately 20.7% ($P<0.01$, Fig. 4A). Moreover, the hepatic absorption of ligustrazine was confirmed to reduce from 196.0±16.5 pmol/106 to 166.0±14.2 pmol/10^6 when quinidine exists, representing a reduction of approximately 15.3% ($P<0.01$, Fig. 4B). (Z)-ligustilide content had no distinct influence by the four transporter inhibitors compared to the control group ($P>0.05$, Fig. 4C). The results indicated that inhibition of organic anion transporter could negatively regulate the hepatic absorption of ferulic acid, while inhibition of organic cation transporter could down-regulate the hepatic absorption of ligustrazine.

(A) The hepatic uptake of ferulic acid after co-incubation with a series of transporter inhibitors. (B) The hepatic uptake of ligustrazine after co-incubation with a series of transporter inhibitors. (C) The hepatic uptake of (Z)-ligustilide after co-incubation with a series of transporter inhibitors. **: $P<0.01$.

Discussion

Chuanxiong Rhizome, a frequently prescribed Chinese herbal medicine in East Asia with a long-standing history of treating cardiovascular and cerebrovascular diseases, as well as acute liver injury and liver fibrosis, has an unclear exposure and uptake mechanism in the liver[19,20]. By focusing on the tissue distribution and transporter- mediated uptake of its main active components, ferulic acid, ligustrazine, and (Z)-ligustilide, we gained valuable insights into its intracorporal processes, which are crucial for understanding its efficacy, safety, drug resistance, and drug-herb interactions.

The liver is the major site for drug metabolism, excretion, and

drug-drug interactions, accounting for approximately 70% of drug elimination in vivo[21]. Our findings have significant implications for potential drug-herb interactions.

Ferulic acid, a representative phenolic acid in Chuanxiong Rhizome, is a substrate for organic anion transporting polypeptide 1B1 (OATP1B1), as evidenced by the significant reduction in its hepatic exposure when organic anion transporter inhibitors like Ritonavir and Rifampin were used. Ritonavir and Rifampin can competitively inhibit the binding of ferulic acid to OATP1B1, reducing its uptake into hepatocytes[22]. In clinical settings, if a patient is taking drugs that are also substrates for OATP1B1 or drugs that can inhibit OATP1B1 (such as certain antiretroviral drugs and antibiotics), the hepatic uptake of ferulic acid may be decreased. This could lead to reduced efficacy of Chuanxiong Rhizome in treating conditions where ferulic acid plays a key role, such as alleviating hepatotoxicity by inhibiting CYP2E1[23].

Ligustrazine, a representative alkaloid ingredient, is likely a substrate for organic cation transporter 1 (OCT1), as indicated by the downregulation of its hepatic absorption when the organic cation transporter inhibitor quinidine was applied. Quinidine can block the function of OCT1, preventing ligustrazine from being transported into hepatocytes[24]. If a patient is taking drugs that are substrates for OCT1 (e.g., some antiarrhythmic drugs) or OCT1 inhibitors, the pharmacokinetics of ligustrazine may be altered, potentially affecting the therapeutic effects of Chuanxiong Rhizome in cardiovascular and cerebrovascular diseases.

Although the mechanism of high uptake of (Z)-ligustilide remains unclear, we speculated that it may not mainly be absorbed into hepatocytes through an active process. One possible explanation is its lipophilicity. Lipophilic compounds have a higher tendency to cross cell membranes via passive diffusion[25]. (Z)-Ligustilide has a relatively high lipophilicity, which may allow it to passively diffuse across the hepatocyte cell membrane[26]. Additionally, its metabolic stability could also contribute to its high hepatic exposure. If (Z)-ligustilide is relatively stable and not rapidly metabolized in the liver, it can accumulate in the hepatocytes over time, leading to high exposure levels.

The variability in herbal medicine use can also be related to our findings. Different sources of Chuanxiong Rhizome may have varying contents of ferulic acid, ligustrazine, and (Z)-ligustilide due to factors such as growing conditions, harvesting time, and processing methods. For example, a batch of Chuanxiong Rhizome grown in a region with specific soil and climate conditions may have a higher content of ferulic acid compared to another batch. This variability in component content can affect the extent of interaction with transporters. A batch with a higher content of ferulic acid may have a more pronounced interaction with drugs that affect OATP1B1 compared to a batch with a lower content.

Moreover, individual differences in transporter expression and function among patients can contribute to the variability in the response to Chuanxiong Rhizome and potential drug-herb interactions. Some patients may have higher or lower levels of OATP1B1 or OCT1 in their livers, which can influence the hepatic uptake and subsequent pharmacokinetics of ferulic acid and ligustrazine. For instance, patients with genetic polymorphisms in the genes encoding these transporters may have altered transporter function, leading to different drug-herb interaction profiles[27].

There are several limitations in our study. Firstly, the sample size in our experiments was relatively small, which may limit the generalizability of our findings. A larger sample size would be needed to confirm the results and draw more robust conclusions. Secondly, we used rat primary hepatocytes in our in vitro experiments. Although rat hepatocytes share some similarities with human hepatocytes, there are also significant differences in transporter expression and function between the two species. Therefore, the results obtained from rat hepatocytes may not fully represent the situation in human hepatocytes, and further studies using human - derived cells or in vivo human studies are necessary. Finally, we did not measure the expression levels of the transporters (such as OATP1B1 and OCT1) in the rat hepatocytes used in our experiments. Without this data, it is difficult to accurately determine the relationship between transporter expression and the uptake of ferulic acid, ligustrazine, and (Z)-ligustilide.

Conclusion

Our study demonstrates that ferulic acid, ligustrazine, and (Z)-ligustilide in Chuanxiong Rhizome extract are highly exposed in the rat liver after oral administration. The high hepatic exposure of ferulic acid and ligustrazine are respectively mediated by organic anion transporters or organic cation transporters.

Declarations

Funding

None.

Conflicts of interest/ Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of Data and Material

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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