Pharmacokinetics of Ligustroflavone in Rats and Tissue Distribution in Mice by UPLC–MS/MS

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An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for quantification of ligustroflavone, which was then applied in pharmacokinetics study in rat and tissue distribution in mouse. Twelve male Sprague Dawley rats were used for pharmacokinetics after intravenous (2 or 8 mg/kg) administration of ligustroflavone, six rats for each dose. Twenty-five mice were randomly divided into 5 groups (5 mice for each group, 1 group for each time point) and received 16 mg/kg ligustroflavone via intraperitoneal administration. The linear range of the calibration curve was over 2–2000 ng/mL for ligustroflavone in rat plasma and mouse tissues. The intra-day and inter-day precision expressed in % RSD were less than 14%, and the accuracy was between 88.5% and 108.4%.

The tissue distribution results indicated that ligustroflavone diffuses rapidly and widely into major organs. The level of ligustroflavone was highest in the mouse liver, followed by the kidney, spleen, and lung. The overwhelming accumulation in the liver indicated that the liver was responsible for the extensive metabolism.

Keywords: mouse, rat, UPLC–MS/MS, ligustroflavone, tissue distribution

Introduction

Fructus Ligustri lucidum is the dried fruit of *Osmanthus plants Ligustrum lucidum* Ait [1–3]. It is used as a traditional Chinese medicine for tonifying the liver and the kidney, has the effect of nourishing the liver and the kidney, and belongs to the top grade of tonifying traditional Chinese medicine [4–6]. *L. lucidum* is rich in Chinese medicinal materials. Modern studies have found that it mainly contains triterpenoids, iridoid terpenoids, flavonoids, and phenylethanoid glycosides, which have the pharmacological effects of regulating immunity, lowering blood glucose, lowering blood lipids, antioxidation, anti-inflammation, bacteriostasis, and protecting liver [7–9]. Ligustroflavone is one of the components with high content of iridoids [10]. It can be used as an exclusive index component of *L. lucidum* for evaluating its quality. Tissue distribution is an indispensable part in the study of pharmacokinetics. It can clarify the change trend of tissue drug concentration with the time of administration, then evaluate the targeting and tissue accumulation of drugs *in vivo*, and judge the safety and effectiveness of drugs.

Liu et al. developed a liquid chromatography–electrospray ionization tandem mass spectrometry method for the qualification of ligustroflavone in rat plasma [11]; however, the tissue study was not reported. A selective and sensitive liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) method was developed for the determination of ligustroflavone in rat plasma and applied to pharmaco-

kinetics after intravenous administration of ligustroflavone (2.0 mg/kg) [11]. Chromatographic separation was performed on a Venusil HILIC column using an isocratic mobile phase consisting of acetonitrile–water–formic acid (75:25:0.1, v/v). The ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method that we developed focused on the analytical ability for ligustroflavone in rat plasma and mice tissues. This method was further applied to the pharmacokinetic analysis after intravenous administration in rat plasma and tissue distribution in mouse, including the heart, liver, spleen, lung, kidney, and brain.

Experimental

Chemicals. Ligustroflavone (purity>98%, Figure 1a) and galangin (internal standard [IS], purity>98%, Figure 1b) were obtained from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, China). Chromatography-grade methanol, acetonitrile, and formic acid were obtained from Merck Company (Darmstadt, Germany). Ultra-pure water (resistance > 18 MΩ) was prepared using a Milli-Q purification system (Bedford, USA).

Instrumentation and Conditions. XEVO TQS-micro triple quadrupole mass spectrometer and ACQUITY H-Class UPLC, equipped with an ESI interface, were from Waters Corp. (Milford, MA, USA). Masslynx 4.1 software (Milford, MA, USA) was used for instrument control and data acquisition.

Chromatographic separation of ligustroflavone and the IS was achieved on an UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with 0.1% formic acid (A) and acetonitrile (B) as the mobile phase. The gradient elution condition was set at the flow rate of 0.4 mL/min: 0–0.2 min (10% B), 0.2–1.5 min (10%–75% B), 1.5–2.0 min (75% B), 2.0–2.5 min (75%–10%

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Figure 1. Chemical structure of ligustroflavone (A) and galangin (IS, B)

B), and 2.5–3.5 min (10% B). The column temperature was set at 40 °C.

The mass parameters were listed as follow: a capillary voltage of 2.0 kV, a desolvation temperature of 400 °C, a desolvation gas (nitrogen) flow rate of 800 L/h, and cone gas flow rate of 50 L/h. MRM modes of m/z 725.3 $\rightarrow$ 271.1 for ligustroflavone and m/z 271.1 $\rightarrow$ 105.1 for the IS were used for quantitative analysis in the positive mode.

Calibration Standards. The stock solutions of ligustroflavone (1.0 mg/mL) and the IS (0.5 mg/mL) were all prepared in methanol. The IS stock solution was further diluted to 0.5 μ g/mL with methanol, which was used as the working solution. The stock solutions and working solutions were stored at 2–8 °C before use.

Ligustroflavone calibration standards were prepared by spiking working solutions of incremental concentration into blank rat plasma. Calibration plots were offset to range between 2–2000 ng/mL for ligustroflavone in rat plasma or tissues (2, 6, 20, 100, 200, 400, 800, 1500, and 2000 ng/mL), each by adding 5 μ L of the appropriate working solution to 50 μ L of blank rat plasma or 50 mg of mouse tissue then mixed by brief vortexing. Quality-control (QC) samples were

prepared in 3 concentrations of rat plasma or mouse tissues (3, 500, and 1800 ng/mL). The proteins of the calibration standards and the QC samples were precipitated using acetonitrile before UPLC–MS/MS analysis.

Sample Preparation. The rat plasma sample and mouse tissues (heart, liver, spleen, lung, kidney, and brain) were prepared according to the methods of Wang et al. (2015) [12].

The 5 μ L IS working solution (0.5 μ g/mL) was added to 50 μ L plasma sample in a 1.5-mL centrifuge tube, and 150 μ L acetonitrile was added, then vortex-mixed for 1.0 min, and centrifuged at 14900g for 10 min; 2 μ L of the supernatant was injected into the UPLC–MS/MS system.

The mouse tissues (heart, liver, spleen, lung, kidney, and brain) were respectively weighed accurately 50 mg and placed in 1.5-mL centrifuge tubes, which were then filled with 200 μ L acetonitrile and 5 μ L IS working solution (0.5 μ g/mL). The mixtures were stored at –80 °C for 10 min, then ground for 3 min by a SCIENTZ-48 Tissue Grinder (64 Hz and 1800 r/s). The tubes were vortex-mixed for 1.0 min and centrifuged at 14900g for 10 min, and 2 μ L of the supernatant was injected into the UPLC–MS/MS system.

Method Validation. Method validation was conducted according to the guidelines set by the European Medicines Agency (EMA) on 3 consecutive days, with 6 replicates of QC samples, and one set of calibration standards each day [13–15]. Validation projects include selectivity, matrix effects, linearity, precision, accuracy, recovery, and stability.

Pharmacokinetic Study. Male Sprague–Dawley rats (200–220 g) were purchased from the Laboratory Animal Center of Wenzhou Medical University. All experimental procedures were ethically approved by the Administration Committee of Experimental Animals of Wenzhou Medical University (Wydw2013–0071). All the rats were fasted for 12 h before the experiment and had free access to water. Blood sample collection from tail vein was performed (0.2 mL) at 0.0333, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 h after intravenous (2 or 8 mg/kg) administration of ligustroflavone, 6 rats for each dose. The blood samples were centrifuged at 3000g for 10 min to harvest plasma, which was then stored at -20°C before further analysis. Plasma ligustroflavone concentration versus time was analyzed by Version 2.0 DAS (Drug and Statistics) software (China Pharmaceutical University).

Tissue Distribution Study. Thirty mice were randomly divided into 6 groups (5 mice for each group, one group for each time point) and received 16 mg/kg of ligustroflavone by intraperitoneal administration. The mice were euthanized by decapitation at 0 (blank group), 0.25, 0.5, 2, 4, and 6 h after administering ligustroflavone. Tissues and blood were removed and washed with saline, and then stored at -20°C .

Results

Method Validation. Figure 2 showed representative extracted ion chromatograms of ligustroflavone and the IS in a blank plasma sample spiked with the analyte. There were no interfering endogenous substances observed which demonstrated the selectivity of the method.

The matrix effects of ligustroflavone were measured at 3 QC concentrations, 94.3%–96.7% ($n = 6$) in rat plasma and 92.5%–110.5% in mouse tissues ($n = 6$). The inter-day and intra-day precision at each QC level was 13% or less and 14% or less, respectively, while the accuracy was between 93.5% and 106.6%. Mean recoveries ranged from 91.0% to 94.6% in rat plasma and 75.5% to 93.9% in mouse tissues. The data above are summarized in Table 1.

Linear regressions were fitted over the 2–2000 ng/mL for ligustroflavone in rat plasma and mouse tissue. The calibration curve was $y = 0.001951x + 0.002410$, $r = 0.9983$ for plasma, $y = 0.000967x - 0.000845$, $r = 0.9979$ for the liver;

Figure 2. Representative UPLC–MS/MS chromatograms of ligustroflavone and galangin (IS) in rat plasma

Table 1. Precision, accuracy, matrix effect, recovery for ligustroflavone of QC sample in rat plasma and mouse tissues ($n = 6$)

Matrix	Concentration (ng/mL)	Precision (CV%)		Accuracy (%)		Matrix effect	Recovery
		Intra-day	Inter-day	Intra-day	Inter-day		
Plasma	3	8.4	3.0	105.2	101.2	96.5	91.0
	500	6.0	2.0	95.5	93.5	94.3	94.6
	1800	6.4	7.5	98.7	106.6	96.7	91.0
Liver	3	10.2	12.3	104.2	95.1	99.2	84.9
	500	8.0	11.8	95.8	95.6	100.8	76.7
	1800	11.0	10.4	97.2	103.4	106.7	82.1
Heart	3	5.5	11.5	94.4	96.1	110.5	90.2
	500	0.3	6.9	99.3	99.8	106.3	93.7
	1800	1.0	7.9	103.9	100.7	107.3	92.9
Lung	3	8.7	10.8	100.7	108.4	101.1	93.9
	500	3.0	12.6	97.8	103.4	107.0	93.2
	1800	9.7	7.0	95.6	105.9	97.8	88.1
Spleen	3	7.0	13.8	99.3	104.8	102.8	83.2
	500	1.0	1.5	95.7	96.1	107.2	80.4
	1800	4.6	1.0	88.5	98.3	105.3	78.8
Kidney	3	12.9	5.8	97.7	95.9	106.2	75.5
	500	2.3	13.6	106.7	106.8	103.2	83.8
	1800	3.3	0.5	98.3	101.7	109.0	81.7
Brain	3	4.2	4.7	108.3	102.0	92.5	77.7
	500	2.9	3.3	95.1	108.3	97.3	78.2
	1800	3.6	3.6	90.9	97.7	96.6	78.6

$y = 0.004203x + 0.004281$, $r = 0.9958$ for the heart; $y = 0.001046x + 0.003664$, $r = 0.9976$ for the spleen; $y = 0.005555x + 0.003068$, $r = 0.9984$ for the lung; $y = 0.001597x + 0.001500$, $r = 0.9982$ for the kidney; and $y = 0.001204x + 0.002478$, $r = 0.9950$ for the brain, where y represents the peak area ratios of ligustroflavone and the IS, and x represents the plasma concentration. The lower limit of quantification (LLOQ) of ligustroflavone in plasma and tissues was 2 ng/mL.

Pharmacokinetic Study. The main pharmacokinetic parameters are listed in Table 2, based on non-compartment model analysis. The plasma concentration–time curves after intravenous (2 and 8 mg/kg) administration of ligustroflavone in rats are shown in Figure 3.

Tissue Distribution. The plasma drug concentration–time curve and tissue distribution results of ligustroflavone in mouse after intraperitoneal administration of 16 mg/kg were respectively shown in Figure 4 and Figure 5, indicating that ligustroflavone diffuses rapidly and widely into major organs. The level of ligustroflavone was highest in the mouse liver, followed by the kidney, lung, and spleen. The level of ligustroflavone in brain remains considerably low, which indicated that the blood brain barrier might effectively prevent ligustroflavone from moving across. The overwhelming accumulation in the liver indicated that the liver was responsible for the extensive metabolism of ligustroflavone.

Discussion

Part of the analytical method was based on our previous study [12] and has been modified as appropriate. A simple

Table 2. Main pharmacokinetic parameters after intravenous administration of ligustroflavone in rats ($n = 6$)

Parameters	Unit	2 mg/kg	8 mg/kg
AUC _(0–t)	ng/mL*h	2458.2 ± 953.6	10047.2 ± 6060.3
AUC _(0–∞)	ng/mL *h	2468.5 ± 953.6	10094.9 ± 6073.4
MRT(0–t)	h	0.9 ± 0.2	1.0 ± 0.5
MRT(0–∞)	h	0.9 ± 0.2	1.0 ± 0.5
$t_{1/2}$	h	1.3 ± 0.5	1.4 ± 0.9
CL	L/h/kg	0.9 ± 0.3	1.0 ± 0.5
V	L/kg	1.8 ± 1.1	2.0 ± 1.7
$C_{\max}$	ng/mL	5226.2 ± 2991.2	14022.6 ± 7536.6

Figure 3. Mean plasma concentration–time profile after intravenous (2 and 8 mg/kg) administration of ligustroflavone in rats

Figure 4. Mean plasma concentration–time profile after intraperitoneal administration of 16 mg/kg ligustroflavone in mice

Figure 5. Mean concentration of ligustroflavone in tissues at different times (0.25, 0.5, 2, 4, and 6 h) after intraperitoneal administration of 16 mg/kg ligustroflavone in mice

protein precipitation method was used in the pretreatment of samples [16–18]. Methanol and acetonitrile were investigated separately. It was found that methanol was easy to introduce

the inherent impurities into plasma, and acetonitrile had higher precipitation efficiency than methanol. The ideal mass spectrometry response could be obtained without concentration and drying process, which greatly saved the analysis time.

Different chromatographic conditions were used to optimize the sensitivity, chromatographic peak type, and analysis speed of the method [19–24]. Specifically, includes different proportion of solvents, such as chromatographic acetonitrile and methanol, and the addition of ammonium acetate, ammonium formate, formic acid, and so on. The addition of formic acid in the mobile phase is conducive to the ionization of the analyte and to obtain a good chromatographic peak. The final mobile phase was acetonitrile–0.1% formic acid, the retention time of ligustroflavone was 1.57 min, and the retention time of galangin was 2.21 min.

Proper internal standard is the key to the analysis of biological samples [25–27]. The selection of internal standard of UPLC–MS/MS should take into account not only the matching of mass spectrum response with the substance to be measured, but also the matching of extraction stability and chromatographic behavior. Several compounds have been screened in the experiment. Galangin was selected as the internal standard, the chromatographic retention was appropriate, and the response was matched.

UPLC–MS/MS for the determination of ligustroflavone in rat plasma was established. The method is sensitive and suitable for the pharmacokinetics and tissue distribution of ligustroflavone. Pharmacokinetic results showed that after intravenous administration, ligustroflavone were eliminated quickly in rats ($t_{1/2}$ of 1.3 ± 0.5 h), and this was consistent with the result ($t_{1/2}$ of 0.88 ± 0.13 h) reported in the literature [11]. In this experiment, the distribution of ligustroflavone in the main tissues was studied. The results showed that ligustroflavone was distributed in the liver, kidney, spleen, lung, heart, and brain of mice. The concentration of ligustroflavone in the brain of mice was low, which may be due to its weak liposolubility and the difficulty of entering the blood brain barrier. According to the concentration of each tissue, the distribution of drug–time histogram was drawn to better understand the metabolism of ligustroflavone in tissues and provide a scientific basis for the broad pharmacodynamic basis of ligustroflavone.

Conclusion

In this study, we presented a simple and sensitive UPLC–MS/MS method for the quantitation of ligustroflavone in 50 μ L of rat plasma or 50 mg of mouse tissue with an LLOQ of 2 ng/mL. We further successfully applied this UPLC–MS/MS method to pharmacokinetic study of ligustroflavone in rat and tissue distribution in mouse. The level of ligustroflavone was highest in the liver, followed by the kidney, spleen, and lung. The considerably low level of ligustroflavone in the brain indicates that ligustroflavone may not cross through blood brain barrier effectively. The distribution of ligustroflavone in mice tissues may contribute to a better understanding of the mechanism of the compound activity in living organisms.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

- Tang, Y. Q.; Li, C.; Sun, X. J.; Liu, Y.; Wang, X. T.; Guo, Y. B.; Wang, L. L.; Ma, R. F.; Niu, J. Z.; Fu, M.; Zhang, D. W.; Li, Y. *BMC Complement. Altern. Med.* **2018**, *18*, 118.

2. Sha, N. N.; Zhao, Y. J.; Zhao, D. F.; Mok, D. K.; Shi, Q.; Wang, Y. J.; Zhang, Y. *Food Funct.* **2017**, *8*, 4703–4712.
3. Chen, B.; Wang, L.; Li, L.; Zhu, R.; Liu, H.; Liu, C.; Ma, R.; Jia, Q.; Zhao, D.; Niu, J.; Fu, M.; Gao, S.; Zhang, D. *Molecules* **2017**, *22*, E1469.
4. Yang, Y.; Nian, H.; Tang, X.; Wang, X.; Liu, R. *J. Ethnopharmacol.* **2017**, *201*, 91–99.
5. Wang, L.; Ma, R.; Guo, Y.; Sun, J.; Liu, H.; Zhu, R.; Liu, C.; Li, J.; Li, L.; Chen, B.; Sun, L.; Tang, J.; Zhao, D.; Mo, F.; Niu, J.; Jiang, G.; Fu, M.; Bromme, D.; Zhang, D.; Gao, S. *Front. Pharmacol.* **2017**, *8*, 266.
6. Tang, X.; Li, X.; Nian, H.; Yang, Y.; Chen, Y.; Wang, X.; Xu, L.; Yang, X.; Liu, R. *Evid. Based Complement. Alternat. Med.* **2017**, *2017*, 7961231.
7. Xu, D.; Lyu, Y.; Chen, X.; Zhu, X.; Feng, J.; Xu, Y. *Mol. Med. Rep.* **2016**, *14*, 4767–4774.
8. Li, Y. P.; Wang, S. J.; Zang, Y. M.; Hu, Z. S.; Liu, C. S. *Chin. J. Nat. Med.* **2016**, *14*, 236–40.
9. Zhang, Y.; Mukwaya, E.; Pan, H.; Li, X. M.; Yang, J. L.; Ge, J.; Wang, H. Y. *Pharm. Biol.* **2015**, *53*, 1082–5.
10. Pieroni, A. Pachaly, P. *Pharmazie* **2000**, *55*, 78–80.
11. Liu, H.; Xu, C.; Wang, W.; Zhao, Y. *J. Chromatogr. Sci.* **2017**, *55*, 267–274.
12. Wang, X.; Wang, S.; Ma, J.; Ye, T.; Lu, M.; Fan, M.; Deng, M.; Hu, L.; Gao, Z. *J. Pharm. Biomed. Anal.* **2015**, *115*, 368–74.
13. Van Toi, P.; Pouplin, T.; Tho, N. D. K.; Phuong, P. N.; Chau, T. T. H.; Thuong Thuong, N. T.; Heemskerk, D.; Hien, T. T.; Thwaites, G. E. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2017**, *1061–1062*, 256–262.
14. Wijma, R. A.; Bahmany, S.; Wilms, E. B.; van Gelder, T.; Mouton, J. W.; Koch, B. C. P. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2017**, *1061–1062*, 263–269.
15. Wang, J.; Zhao, T.; Kong, J.; Peng, H.; Lv, P.; Li, J.; Cao, X.; Zhang, S. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2017**, *1061–1062*, 270–274.
16. Wei, Z.; Ye, L. X.; Jiang, Y. Y.; Zhang, Z. G.; Wang, X. Q. *Lat. Am. J. Pharm.* **2018**, *37*, 523–528.
17. Wang, H. Y.; Chen, Y. C.; Wan, J. F.; Xu, J. L.; Mo, J. J.; Qian, S. Y.; Zhou, Y. F.; Wang, X. Q.; Am, G. Huang. *Lat. J. Pharm.* **2018**, *37*, 1724–1729.
18. Chen, L. G.; You, W. W.; Chen, D. W.; Cai, Y.; Wang, X. Q.; Wen, C. C.; Wu, B. *Biomed. Res. Int.* **2018**, *2018*, 6562309.
19. Wang, X. Q.; Wang, Q. Q.; Hu, Q. P.; Zhao, Y.; Chen, W. H.; Wen, C. C.; Am, B. Wu. *Lat. J. Pharm.* **2017**, *36*, 1403–1407.
20. Chen, D.; Guan, H.; Li, H.; Yang, S.; Liu, Z.; Zhang, L.; Lin, Y.; Wen, C.; Am, X. Yu. *Lat. J. Pharm.* **2015**, *34*, 1913–1919.
21. Chen, D.; Zhang, J.; Peng, X.; Su, K.; Chen, W.; Mo, J.; Wen, C.; Sun, F. *Lat. Am. J. Pharm.* **2016**, *35*, 2279–2283.
22. Ma, J. S.; Wang, S. H.; Huang, X. L.; Geng, P. W.; Wen, C. C.; Zhou, Y. F.; Yu, L. S.; Wang, X. Q. *J. Pharm. Biomed. Anal.* **2015**, *111*, 131–137.
23. Wang, X. Q.; Wang, S. H.; Lin, F. Y.; Zhang, Q. W.; Chen, H. L.; Wang, X. C.; Wen, C. C.; Ma, J. S.; Hu, L. F. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2015**, *983*, 125–131.
24. Zhang, Q. W.; Wen, C. C.; Xiang, Z.; Ma, J. S.; Wang, X. Q. *J. Pharm. Biomed. Anal.* **2014**, *90*, 134–138.
25. Wu, H.; Yan, Q.; Fan, Z.; Huang, M.; He, J.; Ma, J.; Wang, X. *Biomed. Chromatogr.* **2018**, *32*, e4255.
26. Wang, S. H.; Lin, Z. X.; Su, K.; Zhang, J.; Zhang, L. J.; Gao, Z. M.; Wang, Z. Y.; Ma, J. S.; Wang, X. Q. *Acta Chromatogr.* **2018**, *30*, 26–30.
27. Shao, L. J.; Jin, Y.; Fu, H. Y.; Ma, J. S.; Wang, X. Q.; Jin, Y. X.; Wen, C. C. *J. Anal. Methods Chem.* **2018**, *2018*, 9412708.