Determination of Licochalcone A in Rat Plasma by UPLC–MS/MS and Its Pharmacokinetics

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The aim of this study was to establish a rapid, sensitive, and selective ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method to quantify the concentrations of licochalcone A and apply the technique to its pharmacokinetic study. Analytes were separated on an UPLC ethylene bridged hybrid (BEH) C18 column (2.1 mm × 50 mm, 1.7 μm). The mobile phase was consisted of acetonitrile and 0.1% formic acid with a flow rate of 0.4 mL/min in a gradient elution mode. Multiple-reaction monitoring (MRM) was carried out in a negative mode for licochalcone A (m/z 337.2 → 119.7) and the internal standard (IS) (m/z 609.0 → 300.9). The linearity of licochalcone A was great from 0.53 to 530 ng/mL. The lower limit of quantification and the lower limit of detection were 0.53 ng/mL and 0.26 ng/mL, respectively. The intra-day precision was less than 14%, and the inter-day precision was no more than 11%. The accuracy was from 91.5% to 113.9%, the recovery was over 90.5%, and the matrix effect was between 84.5% and 89.7%. The results of stability were in an acceptable range. The bioavailability was only 3.3%, exhibiting poor absorption. The developed method was successfully applicable for determining the concentrations of licochalcone A and its pharmacokinetic study.

Keywords: UPLC–MS/MS, licochalcone A, pharmacokinetics, bioavailability

Introduction

Licorice, originates from the roots and rhizomes of Glycyrrhiza uralensis, is one of the most commonly and widely consumed herbs in the world. Licorice was listed for invigorating spleen, detoxification, removing phlegm, and the treatment of asthma, pain, fever cough, and stomach ulcer in the Chinese Pharmacopoeia [1]. Previous investigations have shown that glycyrrhizin and its derivatives in licorice were the main active components responsible for the hepatic protective and anti-ulcer effects of licorice [2]. Licochalcone A, a major natural chalcone derived from licorice, was also reported to have a variety of bioactivities, such as antioxidant, anti-inflammatory, antispasmodic activity, immunomodulatory effects, and antibacterial activities [3–6]. In recent years, licochalcone A have caught more attention of researchers due to its potent anti-cancer activity to different kinds of cancers [7], including cervical cancer [8], lung cancer [9], breast cancer [10], bladder cancer [11], gastric cancer [12], and so on.

In order to further understand the regulation of absorption, distribution, and metabolism, it was important to perform a pharmacokinetic study for clinical practice. Therefore, it was necessary to develop a quantitative method to detect the concentrations of licochalcone A in rat plasma for pharmacokinetic study. Until now, only a few researches for detecting the concentrations of licochalcone A were reported. Previous study showed that the contents of licochalcone A were investigated in ethanolic extracts of plant specimens by a capillary-zone electrophoresis (CZE) method [13] and in biological fluids by a high-performance liquid chromatography (HPLC) method [14], respectively. In addition, Huang et al. investigated the hepatic metabolism of licochalcone A using liquid chromatography–mass spectrometry (LC–MS) method in human liver microsomes [15]. However, to date, the pharmacokinetics of licochalcone A has not been reported.

Compared with capillary-zone electrophoresis, HPLC, and LC–MS, an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was more sensitive, high throughput, and time saving [16–20]. In this study, licochalcone A was quantified using a newly developed and validated UPLC–MS/MS method to evaluate its pharmacokinetics in SD rat plasma after intravenous and gavage administration.

Materials and Methods

Experimental Materials. Licochalcone A (purity: >98%, presented at Figure 1A) and neohesperidin (purity: >98%, presented at Figure 1B) were bought from Chengdu Mansite Biotechnology Co., Ltd. (Chengdu, China). Chromatographic methanol and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Chromatographic formic acid was got from Tedia (Ohio, USA). A Milli-Q system (Bedford, MA, USA) used for providing ultrapure water was needed. Sprague Dawley (SD) rats (male, weight 200–220 g) were provided by Animal Experimental Central of Wenzhou Medical University.

Experimental Device. The UPLC–MS/MS system was consisted of ACQUITY I-Class UPLC and XEVO TQ-S micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) with an electrospray ionization (ESI) source. The output-signal monitoring and processing were performed by Masslynx 4.1 software (Waters Corp.).

Desolvation gas and cone gas were filled with high purity nitrogen with a flow rate of 800 L/h and 50 L/h, respectively.

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The parameters of detector in the negative mode (ESI−) were presented as follows: capillary voltage was set to 2.2 kV, the temperature of the ESI source, and desolvent were separately 150 °C and 400 °C. Multiple reaction monitoring mode was applied in a negative mode for licochalcone A ions at m/z 337.2 → 119.7 (the collision voltage was 15 V, and the cone voltage was 32 V) and IS ions at m/z 609.0 → 300.9 (the collision voltage was 26 V, and the cone voltage was 35 V).

The UPLC analysis was performed on an UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm). The mobile phase contained acetonitrile (ACN) and 0.1% formic acid with a flow rate of 0.4 mL/min. The temperature of column was set at 40 °C. An effective chromatographic separation was achieved in a gradient elution mode with a total time of 4 min. The chromatographic separation was set as follows: ACN kept at 10% for 0.2 min, then changed linearly to 75% in 1.3 min and was maintained at 75% for 0.5 min, subsequently decreased to 10% in 0.5 min, and finally equilibrated again with 10% for 1.5 min.

Preparation of Standard Solutions. The stock solutions of licochalcone A and neohesperidin were made up of methanol (clear glass, cone-shaped with plastic stent). Finally, 2 μL of aliquot liquid was injected into the UPLC−MS/MS system for analysis.

Validation of Methods. Selectivity was estimated by detecting blank plasma samples, blank plasma samples adding licochalcone A and IS and plasma samples from a rat after dosing. Plasma specimens were prepared using the preparation procedure described above.

The calibration curves were measured by analyzing the peak-area ratios of licochalcone A against the corresponding IS versus the nominal concentration (x) of licochalcone A using a least-squares linear regression method. The linearity was investigated at 9 levels covering the concentration ranging from 0.53 to 530 ng/mL. The linearity could be accepted when the correlation coefficient (r²) was over 0.99.

According to the guidelines of the Food and Drug Administration (FDA) [21], the lower limit of detection (LLD) of the targets must achieve requirements that ratios of signal to noise (S/N) were over 3:1. The lower limit of quantification (LLOQ) of targets was calculated as the lowest concentration of quantitative analysis when S/N was at least 10.

Precision was evaluated by determining three different concentrations of QCs (1.06, 95.4, and 424 ng/mL; n = 3) for three consecutive days. Inter-day and intra-day precisions were calculated as RSD value (less than 15%) for validation.

The peak area ratios of the three concentrations of QCs (1.06, 95.4, and 424 ng/mL), which were added before extraction (IS were added after) against those of the corresponding standard samples obtained by adding licochalcone A and IS after the extraction step, were used to evaluate the recovery (n = 6).

The matrix effect was calculated by comparison of the peak areas of licochalcone A in the extracted blank plasma samples at the three concentrations (1.06, 95.4, and 424 ng/mL) with those of the corresponding standard solutions dissolved with ACN and 0.1% formic acid (1:1, v/v) at equivalent concentrations (n = 6).

The stability of licochalcone A in rat plasma was evaluated by comparing the areas of the QC’s at three concentrations (1.06, 95.4, and 424 ng/mL) in the plasma samples (n = 3). Stability experiments were carried out in four different storage conditions, including storage at room temperature for 6 hours, storage at −70 °C for a month, and freeze–thaw conditions (from −70 °C to room temperature, 3 cycles).

Pharmacokinetic Study. Before administration, SD rats were allowed to eat and drink freely and were kept at room temperature with 12 h on and 12 h off light cycle for seven days. Licochalcone A was dissolved in 2% DMSO solution for dosing and freshly prepared before the experiment. All rats (n = 12) were divided into two groups (group A and group B, n = 6 for each group). Group A received licochalcone A intravenously (5 mg/kg), while group B received this compound orally (15 mg/kg licochalcone A). Over 200 μL of the blood specimens were collected from casual vein before dosing and at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, and 12 h after administration and transformed into 1.5 mL Eppendorf (EP) tubes contained heparin. A 50 μL of plasma was acquired after centrifugation at 3000 rpm for 10 min and stored at −20 °C. DSA software (Version 2.0, China Pharmaceutical University, China) was used for calculating pharmacokinetic parameters. Bioavailability was calculated as Absolute bioavailability = 100% × AUICp − Div/(AUICiv − Dpo), where AUICiv and AUICp are the AUC of the drug from 0 to ∞ after intravenous and oral administration. Div and Dpo are the single dosage of licochalcone A for intravenous and oral administrations, respectively.

Figure 1. The chemical structure of licochalcone A (A) and neohesperidin (B)
Results and Discussion

Optimization of Methods. To optimize the MS conditions, the choice of positive and negative modes of the ESI source played an important role in the methodology [22, 23]. Licochalcone A, a kind of flavonoids, was an acidic compound because it contains phenolic hydroxyl groups. Therefore, the negative mode was more suitable for the determination of licochalcone A theoretically. Ultimately, we chose the negative ESI mode for the detection because of the more strong and stable responses of the analytes, as compared to the positive ion mode.

Endogenous compounds in plasma might interfere the determination of analytes; therefore, a suitable mobile phase condition was necessary to isolate these compounds from analytes at same retention times and obtain a more symmetrical peak shape [24, 25]. Four different combinations of mobile phase were investigated in this study, including ACN and 0.1% formic acid, ACN and 10 mmol/L ammonium acetate solutions (0.1% formic acid included), methanol and 0.1% formic acid, and methanol and 10 mmol/L ammonium acetate solutions (0.1% formic acid included). As a result, ACN and 0.1% formic acid was used as the mobile phase in gradient elution mode for less analysis time, sharper chromatographic peak shape, and the most satisfied resolution.

The plasma is a kind of complex biological specimen with a lot of endogenous substances and proteins, and different extraction methods would directly affect the results of quantification [26–31]. Thus, an effective and simple sample preparation is needed to get rid of these substances before UPLC–MS/MS analysis and obtain a superior bioanalysis method [32–37]. Liquid–liquid extraction and direct protein precipitation were two commonly used sample preparation methods. In this current research, analytes were extracted by liquid–liquid extraction with ethyl acetate, chloroform, and ether and by direct protein precipitation with methanol, ACN, the mixture of ACN, and methanol (1:1, v/v). Even though liquid–liquid extraction had a better extraction and matrix effect, protein precipitation by ACN was the best choice in this study for its relatively good recovery and an acceptable matrix effect.

Validation of Methods

Selectivity. Figure 2 presented the typical UPLC–MS/MS mass spectrum of the blank plasma extract spiked with licochalcone A and IS. There was no interference peak observed at the retention time of licochalcone A or IS, showing a great selectivity.

Calibration Curve. The regression equation of calibration curve was $y = 0.0045x + 0.0061$ ($r = 0.9984$) for licochalcone A. Among them, $y$ is the peak area ratio of licochalcone A against IS, $x$ is the concentration of licochalcone A. The linearity of licochalcone A is great in the calibration curves over the concentration range 0.53 to 530 ng/mL in rat plasma. The LLOQ was 0.53 ng/mL, and the LLOD was 0.26 ng/mL.

Precision, Accuracy, Recovery, and Matrix Effect. Table 1 presented the precision, accuracy, recovery, and matrix effect of licochalcone A. Intra-day precision assessed using the relative standard deviation (RSD) was less than 14%, and the inter-day precision (RSD) was no more than 11%. The accuracy ranged from 91.5% to 113.9% at each QC level. All of the recoveries were over 90.5%, and the matrix effects were between 84.5% and 89.7%. These data suggest that the precision, accuracy, recovery, and matrix effect of licochalcone A were in the acceptable range according the guidelines of the FDA, and the established UPLC–MS/MS method met the pharmacokinetic study.

Stability. The results showed that licochalcone A stored at room temperature for 6 h, −70 °C for a month, or after three freeze–thaw cycles was stable (RSDs ≤ 15 % in all stability tests), which indicated a reliable stability behavior of licochalcone A under the different storage conditions.

Pharmacokinetic Study. No adverse reactions were observed in the two groups throughout the experimental process. The validated method was successfully applied to intravenous and oral administration study of licochalcone A in rats. Pharmacokinetics is a discipline that quantitatively investigates the absorption, distribution, and excretion of drug in vivo. The pharmacokinetics of licochalcone A was reported for the first time.

The mean blood concentration–time curves of licochalcone A are shown in Figures 3 and 4, respectively. The main pharmacokinetic parameters after dosing based on non-compartment mode were shown in Table 2. The AUC(0–t) was 2479.9 ± 326.6 ng/mL·h and 243.3 ± 44.4 for intravenous and oral administration. The bioavailability of licochalcone A was only 3.3%. The low

Table 1. Accuracy, precision, matrix effect, and recovery of licochalcone A in SD rat plasma

| Concentration (ng/mL) | Intra-day Accuracy (%) | Inter-day Accuracy (%) | Precision (RSD%) | Intra-day Precision (%) | Inter-day Precision (%) | Matrix effect (%) | Recovery (%) |
|-----------------------|------------------------|------------------------|------------------|-------------------------|-------------------------|-------------------|--------------|
| 0.53                  | 112.9                  | 113.9                  | 13.0             | 10.7                    | 84.5                    | 95.5              |              |
| 1.06                  | 91.5                   | 96.2                   | 4.4              | 2.7                     | 86.8                    | 92.6              |              |
| 95.4                  | 100.9                  | 93.3                   | 7.4              | 9.4                     | 89.7                    | 90.5              |              |
| 424                   | 97.2                   | 102.2                  | 9.2              | 8.8                     | 88.8                    | 95.0              |              |

Figure 2. The MS/MS chromatograms of licochalcone A and neohesperidin (IS): a blank extract with medium chain triglycerides (MCT) and IS

Figure 3. Mean blood concentration of licochalcone A after intravenous administration at the dose of 5 mg/kg
bioavailability after oral administration exhibited a poor absorption and indicated that licochalcone A was easily affected by the first pass effect in the liver (or the intestine).

Conclusion

We developed and validated a novel and selective UPLC–MS/MS method to determine the licochalcone A in SD rat plasma. The application of this method for extracting licochalcone A from plasma by a simple protein precipitation procedure was more convenient and faster than traditional and commonly used analytical techniques. We have successfully applied this method to the pharmacokinetic investigation of licochalcone A in rat after intravenous and intragastric administration. The oral bioavailability of licochalcone A in mice is 3.3%, exhibiting poor absorption.

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Reference

1. Pharmacopoeia, C.o.C. Chinese Pharmacopoeia (Part II) China Chemical Industry Press, Beijing, 2010, vol 1, p. 247.

2. Fu, Y.; Chen, J.; Li, Y. J.; Zhang, Y. F.; Li, P. Food. Chem. 2013, 141, 1063–1071.

3. Jia, T.; Qiao, J.; Guan, D.; Chen, T. Inflammation 2017, 40, 1894–1902.

4. Tsukiyama, R.; Katsura, H.; Tokuriki, N.; Kobayashi, M. Antimicrob. Agents Chemother. 2002, 46, 1226.

5. Fontes, L. B.; Dos Santos Dias, D.; de Carvalho, L. S.; Mesquita, H. L.; da Silva Reis, L.; Dias, A. T.; Da Silva Filho, A. A.; do Amaral Correa, J. O. J. Pharm. Pharmacol. 2014, 66, 886–889.

6. Nagan, H.; He, J. X.; Tani, T.; Aka, T. J. Pharm. Pharmacol. 2007, 59, 1421–1426.

7. Chen, X.; Liu, Z.; Meng, R.; Shi, C.; Gao, N. J. Ethnopharmacol. 2017, 198, 337.

8. Tsai, T. P.; Lee, C. H.; Ying, T. H.; Lin, C. L.; Lin, C. L.; Hsueh, J. T.; Hsieh, Y. H. Oncotarget 2015, 6, 28851–28866.

9. Qiu, C.; Zhang, T.; Zhang, W.; Zhou, L.; Yu, B.; Wang, W.; Yang, Z.; Liu, Z.; Zou, P.; Liang, G. Int. J. Mol. Sci. 2017, 18, 1761.

10. Kang, T. H.; Seo, J. H.; Oh, H.; Yoon, G.; Chae, J. I.; Shim, J. H. J. Cell. Biochem. 2017, 118, 4652–4663.

11. Wang, X.; Jiang, J.; Yang, X.; Han, J.; Zheng, Q. Mol. Med. Rep. 2016, 14, 911–919.

12. Lin, X.; Tian, L.; Wang, L.; Li, W.; Xu, Q.; Xiao, X. Oncol. Lett. 2017, 13, 1595–1601.

13. Rauschensteiner, F.; Matsumura, Y.; Yamamoto, Y.; Sajani, S.; Tani, T. J. Pharm. Biomed. Anal. 2005, 38, 594–600.

14. Nadelmann, E.; Tjelmeland, J.; Christensen, E.; Hansen, S. H. J. Chromatogr. B: Biomed. Sci. Appl. 1997, 695, 389–400.

15. Huang, L.; Nikolic, D.; van Bremen, R. B. Anal. Bioanal. Chem. 2007, 390, 6937–6948.

16. Chen, L. G.; Wang, Z.; Wang, S.; Li; Pan, Y.; Liu, L. J. Chromatogr. B: Anal. Sci. 2016, 54, 1336–1340.

17. Ye, W.; Chen, R.; Sun, W.; Huang, C.; Lin, X.; Dong, Y.; Wen, C.; Wang, X. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2017, 1068, 144–149.

18. Wang, S.; Wu, H.; Huang, X.; Geng, P.; Wen, C.; Ma, J.; Zhou, Y.; Wang, X. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2015, 990, 118–124.

19. Zhang, Q.; Wen, C.; Xiang, Z.; Ma, J.; Wang, X. J. Pharm. Biomed. Anal. 2014, 90, 134–138.

20. Wen, C. C.; Lin, C. L.; Cai, X. J.; Ma, J. S.; Wang, X. Q. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2014, 944, 35–38.

21. U.S. Department of Health and Human Services, Guideline on Bioanalytical Method Validation Guidance for Industry, 2011, https://www.fda.gov/downloads/Drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf.

22. Popowicz, N. D.; O’Halloran, S. J.; Fitzgerald, D.; Lee, Y. C. G.; Joyce, J. M.; Hu, L. F.; Chen, J. M.; Geng, P. W.; Wei, M. H.; Wang, X. Q.; Ma, J. S.; Wang, X. Q.; Huang, X. L.; Liu, Z. Z.; Lin, Y. Y.; Yang, S. P.; Wen, C. C.; Lin, C. L.; Cai, X. J.; Ma, J. S.; Wang, X. Q.; Wei, Z.; Ye, L. X.; Jiang, Y. Y.; Zhang, Z. G.; Wang, X. Q.; Wu, H.; Luo, X. H.; Wang, X. Q. Lat. Am. J. Pharm. 2016, 35, 2261–2266.

23. Jiang, Y.; Zhou, H.; Su, K.; Xu, M. Z.; Chen, B. B.; Chen, D. X.; Wen, C.; Wang, X. Q.; Lu, H.; Liu, Z. Z.; Sun, W.; Huang, C.; Lin, X.; Dong, Y.; Wen, C.; Wang, X. Q. Lat. Am. J. Pharm. 2016, 35, 1340–1347.

24. Kwon, M. H.; Jeong, J. S.; Ryu, J.; Cho, Y. W.; Kang, H. E. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2017, 1068–1069, 289–296.

25. Ren, K.; Qian, S. Y.; Tu, X. T.; Peng, X. F.; Chen, W. H.; Lin, G. T.; Wang, J. F.; Ma, J. S.; Zhang, Z. A.; Wen, C. C.; Wang, Y. L. Lat. Am. J. Pharm. 2017, 36, 1245–1249.

26. Wu, H.; Yan, Q.; Fan, Z.; Huang, M.; He, J.; Ma, J.; Wang, X. Biomed. Chromatogr. 2018, e4255.

27. Wei, Z.; Ye, L. X.; Jiang, Y. Y.; Zhang, Z. G.; Wang, X. Q. Lat. Am. J. Pharm. 2018, 38, 523–528.

28. Wang, S. H.; Lin, Z. X.; Su, K.; Zhang, J.; Zhang, L. J.; Gao, Z. M.; Wang, Y. Y.; Ma, J. S.; Wang, X. Q. Acta Chromatogr. 2018, 36, 2327–2330.

29. Geng, P.; Luo, J.; Weng, Z.; Fan, Z.; Zhang, B.; Ma, J.; Wang, X.; Zhang, M. Biomed. Chromatogr. 2018, e4273.

30. Wang, X. Q.; Wang, Q. Q.; Hu, Q. P.; Zhao, Y.; Chen, W. H.; Wen, C. C.; Wu, B. Lat. Am. J. Pharm. 2017, 36, 1403–1407.

31. Zhang, M. L.; Zhang, J.; Wen, L. Y.; Wu, X. L.; Wen, C. C.; Wang, X. Q. Lat. Am. J. Pharm. 2016, 35, 2327–2330.

32. Wang, S.; Wu, H.; Geng, P.; Lin, Y.; Liu, Z.; Zhang, L.; Ma, J.; Zhou, Y.; Wang, X.; Wen, C.; Biomed. Chromatogr. 2016, 30, 1145–1149.

33. Wen, C. C.; Wang, S. H.; Huang, X. L.; Liu, Z. Z.; Lin, Y. Y.; Yang, S. P.; Ma, J. S.; Zhou, Y. P.; Wang, X. Q. Biomed. Chromatogr. 2015, 29, 1809–1810.

34. Wang, Q. X.; Wang, S. H.; Ma, J. S.; Ye, T.; Lu, M.; Fan, M.; Deng, M.; Hu, L. F.; Gao, Z. J. Pharm. Biomed. Anal. 2015, 115, 368–374.

35. Wang, X. Q.; Ding, T.; Chen, J. M.; Geng, P. W.; Wei, M. H.; Wang, X. Q.; Zhou, Y. F. Lat. Am. J. Pharm. 2015, 34, 253–258.

36. Tian, W. Q.; Cai, J. Z.; Xu, Y. Y.; Luo, X. H.; Zhang, J.; Zhang, Z. X.; Zhang, Q. W.; Wang, X. Q.; Wang, X. Q.; Hu, L. F.; Lin, G. Y. Int. J. Clin. Exp. Med. 2015, 8, 1516–1517.

37. 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.

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