Determination of coumarin-3-carboxylic acid in the liver and heart tissue of Sprague-Dawley rats after intragastric administration of extractive of leaves of F. virens var. sublanceolata by HPLC

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ABSTRACT

The study was aimed to validate and optimize high performance liquid chromatographic (HPLC) method for the determination of coumarin-3-carboxylic acid (C3A) in the heart and liver issue of Sprague-Dawley (SD) rats after intragastric administration of extractive of leaves of Ficus virens var. sublanceolata. And simple ADME and target prediction analyses were performed for C3A. Ethyl acetate was employed to precipitate protein with appropriate sensitivity and acceptable matrix effects. The satisfactory separation was developed on an ODS2 column (4.6 mm × 250 mm, 5 μm) by gradient elution with a methanol-acetic acid solution (pH = 3.0) as the mobile phase. The flow rate was 1.0 mL min⁻¹, the column temperature was maintained at 30 ± 2 °C, the injection volume was 20 μL, and the detection wavelength was set as 309 nm. The method was fully validated in terms of selectivity, linearity, accuracy, precision, extraction recovery and stability. The results of the ADME analysis found that C3A has excellent characteristics of drug-likeness, consistent with good bio-absorption. And the predicted 12 target protein belongs to the amine oxidoreductase and carbonic anhydrase target class. This method is simple, rapid, sensitive, and accurate for the determination of coumarin-3-carboxylic acid in the heart and liver tissue of SD rats.

KEYWORDS

leaves of F. virens var. sublanceolata, total flavonoids extraction, coumarin-3-carboxylic acid, HPLC, liver tissue, heart tissue

INTRODUCTION

Ficus virens var. sublanceolata (FVS) [1] is a tall deciduous tree plant of the genus Ficus in the family Moraceae, also known as Ficus Grandiflora and Ficus Yellow joint. As a Ficus species, the leaves and roots of FVS can be used as medicine. The literature has reported that the main components are flavonoids, triterpenoids, polyphenols, and tannins [2].

Currently, little attention has been paid to FVS in China, with minority studies focusing on the extraction methods and pharmacological effects (cellular level) of flavonoid components of FVS leaf [3–5]. Because of the wide distribution of FVS and the advantages of flavonoid components with wide clinical applications, such as analgesic, anti-bacterial, anti-inflammatory, anti-stress, anti-allergic, anti-atherosclerosis, and anti-diabetic [6–8], it is of high research value to develop new drugs using flavonoids from the FVS leaf. Flavonoids are important in preventing and treating cardiovascular and cerebrovascular diseases [9] and...
liver diseases [10], so it seems necessary to corroborate some issues by determining the content in the liver and heart tissue. Later, coumarin-3-carboxylic acid (C3A), an important component of total flavonoids, was brought to our attention. C3A has some important applications. For example, the combination of valproic acid with C3A suppresses the proliferation and migration of lung cancer cells [11]. Thus, the determination of C3A in the liver and heart tissue will help follow up further studies of flavonoid components in the leaves of FVS.

In the present study, the total flavonoids in the leaves of FVS were extracted by the heating reflux method [12]. A high-performance liquid chromatographic method was established for determining coumarin-3-carboxylic acid in the heart and liver tissue of SD rats. Combined with the analysis of ADME properties and target prediction of C3A, which will lay a foundation for further studies on the biological activity and provide greater possibilities for developing new drugs based on extractive of leaves of FVS.

MATERIALS AND METHODS

Chemicals and reagents

The leaves of FVS were extracted from the campus of Chongqing Medical University and identified as leaves of FVS, family Moraceae, by Pharmacognosy teaching and Research Office, Chongqing Medical University.

The chemical reference substance of C3A (purity: >98%) was kindly purchased from Shanghai Maclean Biochemical Technology Co., Ltd. (Shanghai, China). Ferulic acid (purity: >98%) was acquired from Chengdu Pfd Biological Co., Ltd. (Chengdu, China). HPLC grade methanol was purchased from TEDIA (USA). Phosphoric acid was purchased from Chongqing Chuandong Chemical Co., Ltd. (Chongqing, China). Acetic acid was purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). Sodium heparin was purchased from Beijing Suolabao Technology Co., Ltd. (Beijing, China). Ethyl acetate was purchased from Shanghai Maclean Biochemical Technology Co., Ltd. (Chengdu, China).

Instruments and conditions

A Waters e2665 HPLC analysis system equipped with a 2489 UV-Vis detector (Waters, USA). The chromatographic separation and analysis were achieved on an ODS2 column (4.6 mm × 250 mm, 5 μm) (Agilent Technologies Co., Ltd.), with the column temperature at 30 ± 2 °C.

The mobile phase was composed of the methanol (eluent A) and acetic acid solution (pH = 3) (eluent B), with a gradient elution as follows: 91%–60% B from 0 to 25 min, 60%–15% B from 25 to 40 min, 15% B from 40 to 55 min, 15%–91% B from 55 to 57 min, 91% B from 57 to 72 min. The injection volume and the flow rate were set at 20 μL and 1.0 mL min⁻¹, respectively. The detection wavelength was 309 nm. Equally valid is this: all mobile phases need to be filtered for impurities and degassed before use.

Preparation of standard solution

Weigh 10.0 mg of coumarin-3-carboxylic acid (C3A) and 5.0 mg of ferulic acid (internal standard), respectively, in a volumetric flask (10 mL). Add methanol to dissolve and fix the volume to the scale, and shake well to obtain a stock solution of coumarin-3-carboxylic acid at a concentration of 1.0 mg mL⁻¹ and ferulic acid at a concentration of 0.5 mg mL⁻¹. Dispense into EP centrifuge tubes and store in the refrigerator at −20 °C. When ready for use, the solution was thawed step by step and diluted to the target concentration with the methanol solution.

Preparation of extractive for intragastric administration

As the heating reflux method mentioned in the introduction section, the specific preparation conditions were as follows: 95 °C water bath extraction temperature; A material-solvent ratio of 1:50 (g: mL); Extraction time of 2 h and solvent of 60% ethanol solution. The extractive of leaves of FVS was concentrated to one-thirtieth of the original volume using a rotary evaporator. Thus, 1 mL of the concentrate contains about 0.6 g of dried powder of the leaves of FVS.

Preparation of heart and liver tissue

SD rats, three males and three females weighing 180–220 g, were supplied by the Animal Laboratory Center, Chongqing Medical University (Chongqing, China). SD rats were given FVS leaf extract concentrate at a dose of 6.0 g kg⁻¹ by intragastric administration; fasting was started 12 h before the administration and started timing after the administration. All the protocols and procedures for animal handling were carried out following the guidelines for using laboratory animals. They were approved by the Animal Experimentation Ethics Committee of the Chongqing Medical University (Chongqing, China).

SD rats were anesthetized in an anesthetic tank containing ether cotton and executed. After an autopsy, the heart and liver tissues were removed, placed in saline, and washed well. The washed heart and liver tissue were placed in an EP tube with sodium heparin, weighed, and homogenized by adding an appropriate amount of water. The homogenate was stored in a refrigerator at −80 °C and prepared for testing.

Bio-sample pretreatment

Quality control samples. The heart and liver homogenate stored at −80 °C was removed and thawed in a graded manner. C3A (1.0 mg mL⁻¹) and ferulic acid (0.5 mg mL⁻¹) were added to an aliquot of 300 μL of the heart homogenate in a 4 mL EP tube and vortex for 30 s. Then an aliquot of 1 mL of ethyl acetate was added to precipitate proteins or extract. The mixture was vortexed for 1 min and centrifuged at 13,000 rpm for 10 min to obtain the supernatant, which was collected and evaporated in a reduced-pressure drying oven (35 °C). 60 mL of methanol was added to the residua above and centrifugation at 13,000 rpm for 10 min to obtain the
supernatant that was passed through a 0.22 μm Millipore filter before injection into the HPLC system for analysis. Liver tissue was treated in the same way.

**Samples after intragastric administration.** The heart homogenate stored at −80 °C was removed and thawed in a graded manner. Take an aliquot of 300 μL of the heart homogenate in a 4 mL EP tube, add the appropriate amount of the internal standard (ferulic acid), and vortex for 30s. The subsequent procedure is the same as the pretreatment of quality control samples.

**Method validation**
Method validation was performed according to the guidelines for bioanalytical method validation. The analytical method used in this research was validated to demonstrate its specificity, linearity, the limit of quantification (LOQ), the limit of detection (LOD), precision, accuracy, stability and extraction recovery.

**ADME properties and target prediction**
The molecular file of C3A was downloaded from ChemicalBook (https://www.chemicalbook.com/ProductIndex.aspx) and was saved in mol2 format. ADME properties of C3A are predicted by SwissADME (http://www.swissadme.ch). Then the targets of these compounds were obtained from SwissTargetPrediction (http://www.swisstargetprediction.ch). To improve the reliability of the target predicted results, a protein with a high probability score (>0.5) was selected.

**RESULTS AND DISCUSSION**

**Chromatographic conditions optimization**
In this experiment, the mobile phase systems of methanol-phosphoric acid solution (pH = 3) were investigated to establish chromatographic conditions. The phosphoric acid solution was first attempted as the inorganic phase. Due to the viscosity of the phosphoric acid solution, the one-way valve of the HPLC was easily blocked, which occurred several times during the experiment. The chromatographic conditions were optimized by changing the mobile phase composition and using a less concentrated acetic acid solution. The separation of C3A acid and ferulic acid was still found to be good, and no further blockage of the one-way valve occurred. The retention time of C3A and ferulic acid in the chromatogram of standard solution were 23.832 and 25.832 min, respectively. The system efficiency parameters in the chromatogram of standard solution were as follows: a number of the theoretical plates (N), 65,059 and 62,004; the value of the asymmetry factor (As), 1.316 and 1.028.

**Assay validation**

**Specificity.** The sample was analyzed according to the chromatographic conditions in the section "Instruments and conditions". In this experiment, the specificity was investigated by analyzing the chromatograms of solvent (methanol), blank heart tissue, blank liver tissue, C3A spiked with an internal standard solution, quality control samples, FVS leaves solution spiked with an internal standard, FVS leaves solution. The results are shown in Fig. 1, and Fig. 2, the separation and peak shape of the two peaks are good in the mixture of C3A, and internal standard. There were no interfering peaks for C3A, and the internal standard in methanol, blank tissue, quality control sample, and each substance in the extract of FVS leaves. It indicates that the developed method can be used to determine the content of coumarin-3-carboxylic acid in the heart and liver tissue with good specificity.

**Linearity range.** The coumarin-3-carboxylic acid stock solution was added to the rat blank heart tissue homogenate and diluted step by step to make 7 concentrations of coumarin-3-carboxylic acid samples ranging from 0.5 to 10 μg mL⁻¹. The standard curve of coumarin-3-carboxylic

![Fig. 1. Chromatogram of (A) FVS leaves solution, (B) C3A spiked with IS, (C) FVS leaves solution spiked with IS, (D) blank heart tissue, (E) QCs, (F) methanol
1: Coumarin-3-carboxylic acid; 2: Ferulic acid.](image-url)
acid in heart tissue was obtained by using the mass concentration of C3A as the horizontal coordinate and the ratio of the peak area of C3A to the internal standard (ferulic acid) as the vertical coordinate. Liver tissue was treated in the same way. The results are shown in Table 1, indicating that C3A in the heart and liver tissue had an excellent linear relationship in the measured concentration range (0.5–10 μg mL⁻¹).

Limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined by the signal-to-noise ratio (S/N) method. The concentration of the analyte with an S/N value of 3 is taken as LOD, and the concentration of the analyte with an S/N value of 10 is taken as LOQ. The LOD of C3A in the heart tissue is 0.90 μg mL⁻¹, and the LOQ is 3.01 μg mL⁻¹. The LOD of C3A in the liver tissue is 1.19 μg mL⁻¹, and the LOQ is 3.94 μg mL⁻¹. This result indicated that the method sensitivity was good and could complete the determination of coumarin-3-carboxylic acid in the heart and liver tissue.

Precision. The precision was investigated by intra-day and inter-day relative standard deviation (RSD). Quality control samples (heart tissue) of the low, medium, and high concentrations (1, 4, and 8 μg mL⁻¹) were prepared, and 5 parallel samples of each concentration were examined for 3 consecutive days. Quality control samples (liver tissue) of the low, medium, and high concentrations (0.5, 2, and 10 μg mL⁻¹) were prepared, and 5 parallel samples of each concentration were examined for 3 consecutive days. The results are shown in Table 2, indicating that the method has good precision in the heart and liver tissue.

Accuracy. The quality control samples of the low, medium, and high concentrations were prepared, and 5 parallel samples of each concentration. The accuracy was indicated by calculating the relative recoveries of each concentration. The results showed that the recoveries of coumarin-3-carboxylic acid in the heart tissue were 111.61%, 97.50%, and 98.30%, and the recoveries in the liver tissue were 105.38%, 94.76%, and 86.28%, and the experimental results indicated that accuracy of this method met the requirements.

Extraction recovery. The extraction recoveries were calculated by comparing the peak areas of C3A in the quality control samples after pretreatment with the peak areas of an equal concentration of the control solution. Five parallel quality control samples were utilized in the extraction recovery experiments. The results showed that the extraction recovery of coumarin-3-carboxylic acid in heart tissue was higher than 80.81%, and the extraction recovery in liver tissue was higher than 79.11%, which could meet the requirements for the quantitative analysis of biological samples.

Stability. The stability was determined on the day of treatment and after 3 days at −20°C after pretreatment of

| Analyte | Tissue | Concentration (μg mL⁻¹) | Intra-day precision (RSD, %) | Inter-day precision (RSD, %) |
|---------|--------|------------------------|-----------------------------|-----------------------------|
| C3A     | heart  | 1                      | 6.69                        | 8.67                        |
|         |        | 4                      | 7.22                        | 7.71                        |
|         |        | 8                      | 6.29                        | 6.58                        |
|         | liver  | 0.5                    | 5.35                        | 5.51                        |
|         |        | 2                      | 5.88                        | 5.96                        |
|         |        | 10                     | 7.25                        | 7.35                        |

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**Table 1.** Calibration curves measured for C3A

| Analyte | Tissue | Calibration range (μg mL⁻¹) | Linear equation | r   |
|---------|--------|-----------------------------|-----------------|-----|
| C3A     | heart  | 0.5–10                      | Y = 0.0062X−0.0013 | 0.9992 |
|         | liver  |                             | Y = 0.002X−0.0008  | 0.9986 |

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**Fig. 2.** Chromatogram of (A) blank liver tissue, (B) methanol, (C) QCs, (D) C3A spiked with IS, (E) FVS leaves solution

1: Coumarin-3-carboxylic acid; 2: Ferulic acid.
quality control heart samples \((n=5)\). The results showed that the RSD of the measured values on the first day were 6.69%, 7.22%, and 6.29%, and the RSDs of the measured values after three days were 9.67%, 8.61%, and 7.96%. The same method was applied to liver tissue, and stability results showed that the RSDs of the measured values on the first day were 5.35%, 5.88%, and 7.25%; and the RSDs of the measured values after three days were 5.51%, 6.70%, and 8.66%. We can conclude that the sample was stable over 3 days.

**Determination of tissue samples after intragastric administration**

The SD rats were administered the extractive of FVS leaves. One hour later, the rats were executed according to this method in the section "Preparation of heart and liver tissue", and the heart tissues were removed. The heart tissues were weighed, homogenized, added with internal standard (ferulic acid), treated according to this method in the section "Bio-sample pretreatment", and then injected into the samples for determination. The results showed that the average concentration of C3A in the heart tissues of SD rats was 0.735 \(\mu g\)/g. The same method was used to obtain liver tissue, and the results showed that the average concentration of C3A in the liver tissues of SD rats was 1.8 \(\mu g\)/g.

Obviously, our actual measurement results demonstrate that the C3A content in liver tissue was significantly higher than that in heart tissue. Therefore, we speculate that the role of C3A in liver diseases deserves more attention and further pharmacological experiments for confirmation.

ABME properties and target prediction

It is commonly known that drug-likeness is a vital factor for further evaluation of small bioactive molecules. The assessment results of SwissADME prediction showed that C3A satisfied Lipinski’s rule (0 violation), having excellent characteristics of drug-likeness, consistent with good bio-absorption (bioavailability score = 0.85). More details are shown in Table 3.

In total, 12 targets (probability>0.5) were revealed corresponding to the selected ingredients (C3A), including MAOB, CA7, CA1, CA3, CA12, CA14, CA9, CA4, CA5A, CA2, CA13, and CA5B. From Table 4, we can obtain more information about the predicted targets. It is worth mentioning that the above-mentioned target protein belongs to the amine oxidoreductase and carbonic anhydrase target class and has a wide range of research. For example, monoamine oxidase B (MAOB) is the enzyme that regulates the degradation of dopamine [13] and plays a pivotal role in the metabolism of monoamines [14]. Carbonic anhydrase is a key enzyme that mediates the reversible hydration of carbon dioxide. CA3 has an antioxidative function and plays a crucial role in the maintenance of intracellular pH homeostasis [15]. CA9 is a cancer-related cell surface enzyme catalyzing the reversible conversion of carbon dioxide to bicarbonate ion and proton [16].

**Table 3. ADME properties of C3A predicted by Swiss ADME**

| MW  | TPSA | Log S (ESOL) | GI absorption | BBB permeant | CYP1A2 inhibitor | CYP2C9 inhibitor | Lipinski | Bioavailability Score |
|-----|------|-------------|---------------|--------------|------------------|-----------------|----------|-----------------------|
| 190.15 | 67.51 | −2.87 | High | Yes | Yes | No | 0 | 0.85 |

MW: Molecular weight.
TPSA: Topological Polar Surface Area.

**Table 4. Target prediction of C3A from SwissTarget Prediction**

| Target | Common name | Uniprot ID | Target Class | Probability |
|--------|-------------|------------|--------------|-------------|
| Monoamine oxidase B | MAOB | P27338 | Oxidoreductase | 0.9888 |
| Carbonic anhydrase VII | CA7 | P43166 | Lyase | 0.9888 |
| Carbonic anhydrase I | CA1 | P00915 | Lyase | 0.9888 |
| Carbonic anhydrase III | CA3 | P07451 | Lyase | 0.9888 |
| Carbonic anhydrase XII | CA12 | O43570 | Lyase | 0.9888 |
| Carbonic anhydrase XIV | CA14 | Q9ULX7 | Lyase | 0.9888 |
| Carbonic anhydrase IX | CA9 | Q16790 | Lyase | 0.9888 |
| Carbonic anhydrase IV | CA4 | P22748 | Lyase | 0.9888 |
| Carbonic anhydrase VA | CA5A | P35218 | Lyase | 0.9888 |
| Carbonic anhydrase II | CA2 | P00918 | Lyase | 0.6230 |
| Carbonic anhydrase XIII (by homology) | CA13 | Q8N1Q1 | Lyase | 0.6230 |
| Carbonic anhydrase VB | CA5B | Q9Y2D0 | Lyase | 0.6230 |
CONCLUSION

We established a high-performance liquid chromatographic method for determining C3A in the heart and liver tissues. The method was validated by selecting ferulic acid as the internal standard and using liquid-liquid extraction as a pretreatment method for biological samples. The experimental results showed that the specificity, sensitivity, accuracy and precision met the requirements. And the method could be used to determine C3A in the heart and liver tissues of SD rats after oral FVS leaf extract. The successful establishment of the method with a combination of the results of ADME analysis and target prediction is an essential part of drug discovery and development of flavonoids based on extractive of leaves of FVS. It can also provide a basis for further pharmacological research on leaves of FVS.

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REFERENCES

1. Chen, F. H. Guangdong Flora Volume I; Guangdong Science and Technology Press: China, 1987; pp 186–214.
2. Fan, M. S.; Ye, G.; Huang, C. G. Nat. Prod. Res. Dev. 2005, 17, 497–504.
3. Wang, Y.; Hu, K.; Chen, L.; Su, Y.; Ma, J. H.; Xiao, H.; Bai, Q. H. Chongqing Med. 2017, 46, 2178–82.
4. Dong, L. L.; Lv, J.; He, Y.; Zhou, S. M.; Bai, Q. H.; Xiao, H. Appl. Chem. Ind. (Xi’an, China) 2017, 46, 2188–2190–2194.
5. Lai, F.; Li, Q. Y.; Yang, R. H.; Gao, J. Y.; Xiao, H.; Jia, Y.; Wang, Y.; Bai, Q. H. Chongqing Med. 2019, 48, 554–558–563.
6. Gupta, D.; Guliani, E. Biointerface Res. Appl. Chem. 2022, 12, 5983–95.
7. Zhang, X. M.; Wang, Y. Y.; Wang, H. J. Guangdong Chem. Ind. 2020, 47, 597–608.
8. Qi, J. H.; Dong, F. X. J. Beijing Union Univ. 2020, 34, 89–92.
9. Li, H.; Bai, L.; Qin, Q.; Feng, B. L.; Zhang, L.; Wei, F. Y.; Yang, X. F. Chin. J. Chin. Mater. Med. 2020, 45, 2827–34.
10. Guo, S.; Ren, X.; Du, J.; Zhang, S.; Wang, T.; Zhang, H.; Zhao, H.; Yue, W.; Ho, C. T.; Bai, N. J. Food Biochem. 2020, 44, e13259.
11. Liu, X.; Chen, L.; Sun, F.; Zhang, G. Cytotechnology 2013, 65, 597–608.
12. Liu, Y. H. Study on the Extraction Technology and Metabolism of the Active Components in the Leaves of F. Virens Var Sublanceolata; Chongqing Medical University: Chongqing, 2020; pp 16.
13. Leko, M. B.; Perković, M. N.; Klepac, N.; Strac, D. S.; Borovečki, F.; Pivac, N.; Hof, P. R.; Simić, G. J. Alzheimer’s Dis. 2020, 73, 135–45.
14. Ziegler, C.; Domschke, K. J. Neural Transm. 2018, 125, 1581–8.
15. Dowling, P.; Gargan, S.; Zweyer, M.; Sabir, H.; Swandulla, D.; Ohlendieck, K. Expert Rev. Proteomics 2021, 18, 1073–86.
16. Pastorek, J.; Pastorekova, S. Semin. Cancer Biol. 2015, 31, 52–64.