

## Research Article

# A Validated Liquid Chromatographic Method for Berberine Analysis in Tissue and Application

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Simple and rapid high-performance liquid chromatography methods were developed for the determination of berberine (BB) in various rat tissues so as to evaluate a P-gp inhibitor, glycyrrhetic acid (GA), on BB's oral bioavailability. Acetonitrile was used to extract BB from tissues and showed different extraction recoveries in diverse tissues. The intra- and interday precision and accuracy were less than 10%. Long-term stability, pre (post) -preparative stability, and freeze-thaw stability were evaluated, and the results showed it could meet the need of this study. The proposed methods were subsequently applied to investigate the possible drug-drug interaction of GA and BB in vivo from the aspect of tissue distribution. The results showed that no significant difference was found between the group of low dose and high dose at the same time point. The tissue distributions show a saturated model, i.e., the content of BB in tissue tends to be constant while its dose is more than 200 mg/kg. Besides, the contents of BB ranged from high to low according to the order of the liver, kidney, and spleen. The BB content in the liver is especially high as compared to other tissues.

## 1. Introduction

Berberine (BB) is an isoquinoline alkaloid, which is also the main ingredient of some traditional Chinese medicines (TCMs), such as *Phellodendri Cortex*, *Coptidis Rhizoma*, and *Radix Berberidis* [1, 2]. Glycyrrhizic acid (GL), a triterpenoid, is one of the characteristic ingredients of licorice which is the most commonly used TCM in prescription. Glycyrrhetic acid (GA), namely, the metabolite of GL, is the mainly detected form in plasma during oral administration of GL or Licorice [3, 4]. Moreover, as main ingredients of plant medicine, GA and BB have been found to have various pharmacological activities. For GA, anti-inflammatory [5, 6], antiviral [7], hepatotoxicity protection [8], antiulcer [9], antitumor [10, 11], and adrenal cortical hormone kind function [12] have been reported. Clinical trials have clearly shown that GA has a good effect on all types of dermatitis [13] and purulent scar disease [14]. As to BB, antibacterial [15], antitumor [16, 17], antioxidant [17, 18],

anti-inflammatory [19], and cholesterol-lowering [20–22] effects have been documented. BB has been proved its potential in future clinic application for hyperlipidemia [21], diabetes [24], and cardiovascular [23] and neuroprotective diseases [25].

In prescription of TCM, licorice may combine with *Coptis chinensis* Franch [26, 27], which is now commonly utilized for the treatment of general pyrexia, diabetes mellitus, hyperlipidemia, and diarrhea in clinics [28]. It had been reported that GA could react with BB (acid-base reaction) and produce a precipitate in vitro [29]. This may exert an effect on the bioavailability of BB and/or GA when both of these components exist in the same prescription. Besides, GA has been believed to play a synergistic role with other ingredients in prescription of TCMs during treatment. Moreover GA is reported to be an inhibitor of P-glycoprotein and multidrug resistance protein 1 [30] and BB is also a substrate of P-gp [31]. However, there are no statistics available to address the effects of GA on the tissue

distribution of BB in vivo. Thus, the present study is focused on the effect of oral administration of GA on the tissue distribution of BB in an animal model.

There are some methods for analysis of BB in biological samples, such as capillary electrophoresis [32, 33], a flow-injection chemiluminescence system [34], near-infrared spectroscopy [35], the UPLC-MS/MS method [36, 37], and HPLC with MS/MS [38–43] or a UV detector [44, 45]. These methods focus on the fecal sample [32], plasma [33, 36–43], and herbal or herbal preparations [34, 35, 44, 45]. A few methods pay close attention to the determination of BB in tissues [46–48], in which some needed a two-step extraction [46, 47] and one used the HPLC/MS/MS system [48]. Besides, the HPLC method is a very robust and reliable approach which is the main way for the analysis of constituents in TCMs in the China standard system. Moreover, a cheap and simple method is necessary for the evaluation of drug-drug interaction for a much complicate system such as TCM from the angle of tissue distribution. In this study, high-performance liquid chromatography methods were developed for the evaluation of the effects of GA on the tissue distribution of BB.

## 2. Materials and Methods

**2.1. Chemicals and Animals.** Berberine bought from Aladdin Chemistry Co., Ltd. (HPLC purity 97%); glycyrrhetic acid was obtained from AR, Fluka Chemical Corp., product of Spain; methanol and acetonitrile (HPLC grade) were obtained from Amethyst Chemicals (China); and sodium carboxymethyl cellulose was obtained from Aladdin Chemistry Co., Ltd. Other reagents were of analytical level. Distilled water was used.

Seventy-two Sprague Dawley (SD, SPF level) rats ( $180 \pm 30$  g), male and female each half, were purchased from the Experimental Animal Center of Guangxi Medical University (Guangxi, China). They were kept under room temperature for 2 days before study.

**2.2. HPLC Conditions.** Chromatography detection was carried out on a CTO-20A-type Shimadzu high-performance liquid chromatograph (HPLC) with an SPD-20A and LC-20AT unit, a CTO-20AC column oven, and a Shimadzu C18 column ( $4.6 \times 250$  mm), using methanol-1% acetic acid (51 : 49) as the mobile phase; the column temperature was set at 31 degrees Celsius; the detection wavelength was 350 nm; the flow rate was 1.0 mL/min; the volume of injection was  $20 \mu\text{L}$ ; and the elution was performed in an isocratic mode.

**2.3. Preparation of Solutions.** Berberine stock solution for the HPLC method was prepared by dissolving BB in methanol to obtain 500 ng/mL. The solution was, then, diluted with methanol to achieve standard working solutions. Quality control (QC) samples:  $100 \mu\text{L}$  three/two level concentration of BB was transferred to a centrifuge tube, and then, the samples were prepared according to the sample preparation procedure. High (low)-dose berberine was prepared by dissolving 2.00 g (1.00 g) BB with or without 0.25 g glycyrrhetic acid in 0.5% sodium carboxymethyl cellulose (SCC) solution in a 50 mL volumetric flask.

**2.4. Tissue Distribution Study.** Rats in half genders were randomly divided into several groups, and each had 6 rats. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals by Yulin Normal University (Grant No. YTUDW20190117). BB solutions were intragastrically administered at a dose of 400 or 200 mg/kg body weight with or without GA. Then tissue samples were collected at 1, 2, and 4 h after administration, using six rats at each time point. The blood on the surface of tissue was washed away with normal saline and, then, dried with filter paper. These samples were stored at  $-20$  degrees until used.

**2.5. Sample Preparation.** The frozen tissue samples were naturally thawed. Then, they were cut into several large pieces with scissors, impurities such as blood clots were carefully removed, and then, they were cut into small pieces and dried with filter paper. 0.20 g of these tissues was put into a centrifuge tube. Then, the stuff was homogenized with a homogenizer (Ningbo Xinzhi Biotechnology Co., Ltd.) by addition of 0.5 mL physiological saline. 2 mL acetonitrile was added in succession and extracted for 2 min by vortex. Then, centrifugation was performed for 5 min at a speed of 12000 r/min. 2 mL of the supernatant was transferred to another centrifuge tube and, then, dried by  $\text{N}_2$  flow under a  $50^\circ\text{C}$  water bath. The dried samples were dissolved in 0.1 mL methanol by 2 min vortex. Then,  $20 \mu\text{L}$  of the supernatant was directly sampled in HPLC analysis after centrifuging for 5 min at a speed of 12000 r/min.

**2.6. Method Validation.** The method was validated using rat tissues from healthy rats following the guidelines of bio-analytical method validation as issued by the FDA center for Drug Evaluation and Research. It was performed from the following aspects: specificity, linearity and sensitivity, precision and accuracy, sample stability, and extraction recovery [37, 43].

**2.7. Data Analysis.** The calibration curves (triplicate for each concentration) and relative standard deviation (RSD) were obtained by WPS Office 2019 (Beijing Jinshan office software Co., Ltd). The statistical analysis of the data of two groups (six rats in each time point) was performed with independent samples *t*-tests using the Statistical Program for Social Sciences (SPSS 17.0 for Windows).

## 3. Results and Discussion

**3.1. Selectivity of HPLC Analysis.** The selectivity of HPLC analysis was evaluated by comparing chromatograms of blank tissue samples prior to drug administration, blank tissue spiked with BB standard solution, and samples after drug administration. A good selectivity can be achieved by carefully selecting the column, mobile phase, and elution mode [36]. A reversed phase column is suitable for most cases. Under this circumstance, we tried methanol and acetonitrile and finally chose methanol-1% acetic acid (51 : 49) as the mobile phase. The typical results of the liver sample are shown in Figure 1. The run time of the HPLC

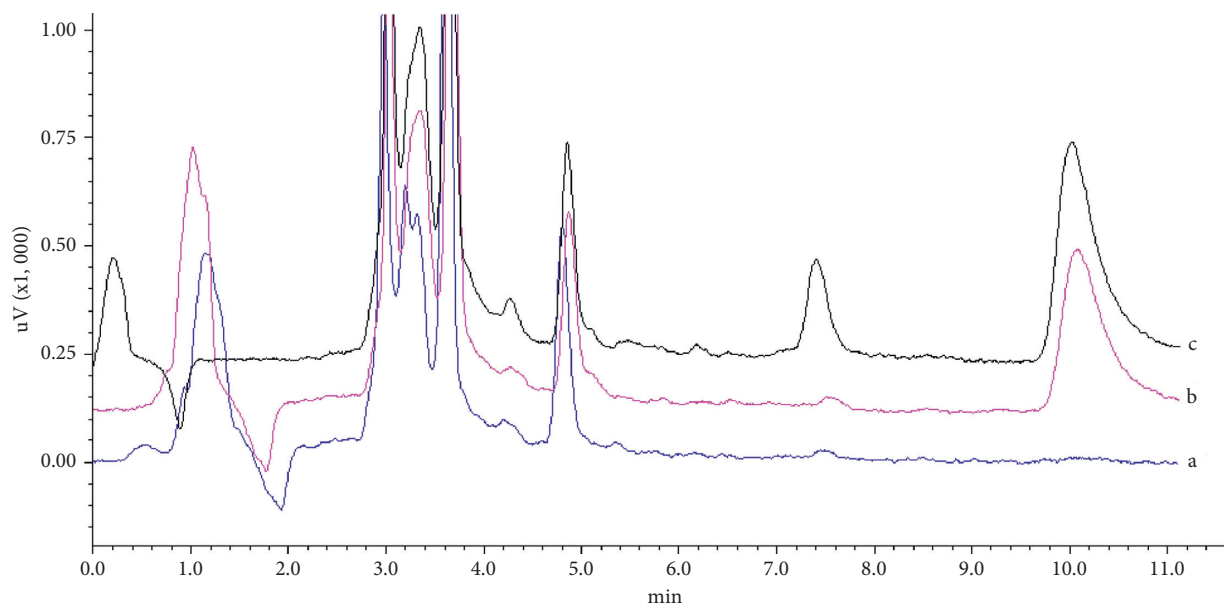


FIGURE 1: HPLC Chromatograms of BB in liver samples. Blank liver sample; (b) blank liver spiked with BB; and (c) liver sample after administration.

method is less than 12 min, and retention time of BB is at 10.19 min. The chromatograms were free of interfering peaks from endogenous substance at the retention time of BB. None of the interfering peaks from the endogenous substance at the retention time of BB is found.

**3.2. Linearity and Sensitivity.** The linear relationships of the methods were evaluated by preparing seven, five, and five different concentrations of samples in the liver, kidney, and spleen using the previous extraction procedure, respectively. Three replications were made for each concentration levels. For the liver, 0.025, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0  $\mu\text{g/g}$  were applied. For the kidney, 0.02, 0.04, 0.10, 0.25, and 0.50  $\mu\text{g/g}$  were applied. For the spleen, 0.025, 0.05, 0.10, 0.25, and 0.50  $\mu\text{g/g}$  were used. The calibration curves were constructed by plotting the peak area of the BB against tissues concentration using the least-squares regression mode. The lower limit of detection (LOD) was the concentration giving a signal-to-noise ratio of, at least, 3-folds. The lower limit of quantitation (LOQ) was the concentration giving a signal-to-noise ratio of, at least, 10-folds. The results are shown in Table 1. Good linearity is found for the liver, spleen, and kidney sample detection. The LOQs basically meet the needs of this study. The LODs and LOQs are equivalent to that reported in the literature [49, 50].

**3.3. Precision and Accuracy.** The precision and accuracy of the method were investigated by determining QC samples at three/two different concentrations (three replicates for each concentration level) over three consecutive days. The precision of the method at each QC concentration was expressed as the relative standard deviation (RSD), and the accuracy was described as recovery (RE). The suitability of the precision and accuracy was assessed by the following criteria: the RSD

should not exceed 10%, and the accuracy should be within  $100 \pm 10\%$  of the actual values for QC samples. The results are presented in Table 2. As can be seen from Table 2, the precisions of the three kinds of samples in three concentration levels are lower than 3.93% and 5.87% (RSD) for within-day and between-day precision, respectively. The precision and accuracy can meet the need of this research.

**3.4. Sample Stability.** Stability of QC samples in tissues was investigated at three concentration levels under different storage conditions: long-term stability at  $-20^\circ\text{C}$  for 2 weeks, pre (post) -preparative stability at room temperature for 4 (8) h, and three freeze-thaw cycles. The stability of samples was expressed as the relative standard deviation (RSD). The results could be found in Table 3. These results show that the samples are stable during the study. We also evaluated a 7-day stability of BB by a diluted stock solution of it. The result showed that the RSD was 1.9%.

**3.5. Extraction Recovery.** Tissue extraction usually includes single-phase extraction and biphasic extraction. The former used a water-soluble solvent, such as methanol and acetonitrile [51], while the latter applied a water-insoluble solvent, for example, ethyl acetate and dichloromethane [46, 47]. The extraction recovery of BB at three or two QC levels was conducted by calculating the ratio of the peak area of blank tissue spiked with BB and the peak area of the standard QC solution. In the liver, the extraction recovery is 74.1%, 72.2%, and 70.3% (average 72.2%) for three QC samples ( $n=3$ ), respectively. In the kidney, the extraction recovery is 63.7% and 59.0% (average 61.4%) for two QC samples ( $n=3$ ), while in the spleen, the extraction recovery is 51.3% and 48.6% (average 50.0%) for two QC samples ( $n=3$ ). The results show that the binding ability of these tissues to BB may be different.

TABLE 1: The calibration curves, LOD, and LOQ of BB in different tissues ( $n = 3$ ).

Samples	Regression equation	Correlation coefficients ( $r$ )	Linear range ( $\mu\text{g/g}$ )	LOD ( $\mu\text{g/g}$ )	LOQ ( $\mu\text{g/g}$ )
Liver	$y = 36033x - 664$	0.9999	0.025–10.0	0.025	0.040
Kidney	$y = 34212x - 380$	0.9996	0.020–0.50	0.017	0.036
Spleen	$y = 31092x - 555$	0.9993	0.025–0.50	0.023	0.035

TABLE 2: Precision and accuracy of the method ( $n = 6$ ).

Tissues	Concentration ( $\mu\text{g/g}$ )	Within-day precision RSD (%)	Between-day precision RSD (%)	Recovery (%)
Liver	0.25	5.30	5.87	92.66
	1.25	2.37	2.53	100.1
	10.0	3.93	4.00	102.2
Kidney	0.10	0.98	1.06	107.0
	0.25	1.74	2.48	92.82
Spleen	0.10	2.82	3.63	106.7
	0.25	1.74	2.32	103.0

TABLE 3: Stability of BB in samples ( $n = 3$ ).

Tissues	Concentration ( $\mu\text{g/g}$ )	Freeze-thaw cycle RSD (%)	Prepreparative RSD (%)	Postpreparative RSD (%)	$-20^\circ\text{C}$ for 2 weeks RSD %
Liver	0.25	4.2	7.5	3.9	9.2
	1.25	3.8	6.4	4.6	9.5
	10.0	3.5	7.1	4.2	9.1
Kidney	0.10	2.1	2.7	1.3	1.1
	0.25	2.4	4.3	2.9	1.9
Spleen	0.10	3.8	4.2	3.3	3.7
	0.25	2.1	2.1	1.6	4.4

TABLE 4: Results of BB at a dose of 400 mg/kg in tissues of rat (mean  $\pm$  SD,  $n = 6$ ,  $\mu\text{g/g}$ ).

Tissue	1 h		2 h		4 h	
	With GA	Without GA	With GA	Without GA	With GA	Without GA
Liver	$0.666 \pm 0.142$	$0.629 \pm 0.267$	$3.21 \pm 1.92$	$2.08 \pm 1.34$	$3.76 \pm 2.73$	$2.42 \pm 2.28$
Spleen	$0.049 \pm 0.012$	$0.062 \pm 0.019$	$0.183 \pm 0.123$	$0.137 \pm 0.109$	$0.349 \pm 0.115$	$0.291 \pm 0.110$
Kidney	$0.195 \pm 0.134$	$0.189 \pm 0.143$	$0.244 \pm 0.120$	$0.267 \pm 0.349$	$0.297 \pm 0.155$	$0.202 \pm 0.064$

TABLE 5: Results of BB at a dose of 200 mg/kg in tissues of rat (mean  $\pm$  SD,  $n = 6$ ,  $\mu\text{g/g}$ ).

Tissue	1 h		2 h		4 h	
	With GA	Without GA	With GA	Without GA	With GA	Without GA
Liver	$0.450 \pm 0.217$	$0.772 \pm 0.343$	$1.814 \pm 2.003$	$3.734 \pm 1.875$	$0.850 \pm 0.268$	$0.882 \pm 0.132$
Spleen	$0.068 \pm 0.037$	$0.0504 \pm 0.023$	$0.114 \pm 0.171$	$0.324 \pm 0.292$	$0.065 \pm 0.060$	$0.075 \pm 0.065$
Kidney	$0.137 \pm 0.148$	$0.397 \pm 0.315$	$0.193 \pm 0.089^*$	$0.287 \pm 0.122^*$	$0.188 \pm 0.052$	$0.241 \pm 0.259$

\*  $p < 0.05$ .

**3.6. Results of Tissues Distribution.** The frozen tissue samples were naturally thawed. 0.20 g of these tissues was taken and treated in accordance with the abovementioned sample processing method. Then, 20  $\mu\text{L}$  volume of the prepared sample was injected for HPLC analysis doubly. The tissue distributions of BB at a dose of 400 or 200 mg/kg body weight with or without GA are shown in Tables 4 and 5. The tissue distributions are presented as microgram BB per gram tissue and calculated by calibration curves. As shown in Tables 4 and 5, no significant difference ( $p > 0.05$ ) is found

except that in the kidney 2 h after a dose of 200 mg/kg between the group with and without GA at the same time point. Moreover, no significant difference ( $p > 0.05$ ) is found between the group of low dose and high dose at the same time point. The tissue distributions show a saturated model, i.e., the content of BB in tissue tends to be constant while its dose is more than 200 mg/kg. Besides, the contents of BB ranged from high to low according to the order of the liver, kidney, and spleen. BB in the liver is especially high as compared to other tissue.



## 4. Conclusions

During preliminary evaluation of the drug-drug in complex system such as TCM, we may judge by determining contents in the late distribution phase, equilibrium phase, and early elimination phase. In this case, where the concentration of components is relatively high, HPLC with a UV detector will achieve good results.

The validated high-performance liquid chromatography methods are simple as to sample preparation. It has good accuracy and precision and has been successfully applied to determine berberine in tissues and evaluate the effect of GA, a P-gp inhibitor, on BB's oral bioavailability. Our study reported, for the first time, the saturated distribution of BB in tissue. The tissue distribution results will provide useful information for the use of berberine in clinical trials.

## Abbreviations

BB: Berberine  
 GA: Glycyrrhetic acid  
 HPLC: High-performance liquid chromatography  
 LOD: The lower limit of detection  
 QC: Quality control  
 TCM: Traditional Chinese medicine.

## Data Availability

The data used to support the findings of this study are included within the article.

## Ethical Approval

All the animal experimental procedures were approved by the animal welfare and Animal Ethics Committee of Yulin Normal University, Yulin, China.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

Dangmei Liu and Xiaowang Bao performed method validation. Neng Zhou designed the project and wrote the original draft, reviewed and edited the paper, and supervised the work.

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