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Determination and tissue distribution comparisons of gentiopicroside after oral administration of raw and wine-Processed *Gentiana Radix*

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Abstract: *Gentiana Radix* is one of the most often used drugs in traditional Chinese medicine. Stir frying with yellow wine is the most common processing method. To clarify the principle of processing, an experiment was carried out to compare the tissue distribution of the typical constituent after oral administration of raw *G. Radix* and wine-processed one. To compare the tissues distribution of gentiopicroside oral administration of raw and wine-processed *G. Radix,* High-performance liquid chromatogram with ultraviolet detection was developed and validated for the determination of gentiopicroside in heart, liver, spleen, lung, kidney, stomach, small intestine and large intestine tissues. The gentiopicroside in raw and wine-processed *G. Radix* was distributed in all tissues involved in this study. Compared with the rats administration of raw *G. Radix*, the proportions of gentiopicroside in heart, liver and lung tissues increased in rats with administration of wine-processed one. The proportion of gentiopicroside in upper-JIAO and liver tissue can be increased by wine-processing.

Keywords: Gentiopicroside, *Gentiana Radix*, raw and wine-processed, tissue distribution.

INTRODUCTION

Gentiana Radix (Chinese name Long Dan) is the dried roots and rhizomes of Gentianaceae plants, such as *Gentiana manshurica* Kitag., *Gentiana scabra* Bge., *Gentiana triflora* Pall. and *Gentiana rigescens* Franch. *G. Radix*, which has the flavor of bitter and cold, belongs to liver and gallbladder meridian. *G. Radix* has the effect of clearing heat and drying dampness, purging liver and gallbladder fire (ChP, 2015). Gentiopicroside is the main active component of *G. Radix*, which has the pharmacological effects of liver protection (Xu *et al.,* 2013), analgesia (Chen *et al.,* 2008), anti-inflammatory (Chen *et al.,* 2008), anti-tumor (Chou *et al.,* 2003), stomachic effect (Ruan *et al.,* 2015), etc. The modern processing method of *G. Radix* was mainly wine-processed. In the traditional theory, processing with wine could moderate the bitter and cold properties of *G. Radix*, while *G. Radix* can get into upper-JIAO and play a part in it. Some scholars have studied the tissue distribution of gentiopicroside in *G. Radix* (Feng *et al.,* 2004), but the difference on the tissue distribution of gentiopiroside between the raw and wine-processed *G. Radix* has not been reported. The present study justified the difference of gentiopicroside in different tissues by comparing tissue distribution between raw and wine-processed *G. Radix*, which can lay the foundation for the rational use of drugs.

MATERIALS AND METHODS

Materials and reagents

G. Radix was purchased from Anguo herbs market in Henan province and was identified as the dry root and rhizome of *Gentiana scabra* Bge. by Pofessor Wang Bing (Liaoning University of TCM). Wine-processed *G. Radix* was made by reference to the Chinese Pharmacopoeia (2015).

Gentiopicroside (purity, 99%) was supplied by the Jiangsu Yongjian Pharmaceutical Technology Company (Nanjing, China). The Internal standard substance called caffeine (purity, 99%) was supplied by the Shanghai Chemical Reagent Company (Shanghai, China). Pure water was supplied by Wahaha Company (Hangzhou, China). HPLC grade methanol was supplied by Kemiou Chemical Reagent Company (Tianjin China). High-speed homogenizer was purchased from Putian Instrument Manufacturing Company (Changzhou, China).

High-performance liquid chromatography condition

An Agilent 1100 series high performance liquid chromatography (four pumps, VWD UV detector, Agilent Technologies Co. Ltd.) was used to quantify the gentiopicroside. The separation of compounds was accomplished by a Welchrom C_{18} analytical column (4.6 mm× 250mm, 5μm, Welch Materials). Flow phase: water - methanol (70: 30, V/V), flow rate: 1.0 ml/min, column temperature: 30°C, injection volume: 20μL.

Preparation of G. Radix solution

75g raw *G. Radix* powder (through NO. 4 sieve) was extracted with 10 times of distilled water by reflux for 1h, then filtered while it was still hot. The residue was extracted with 8 times of distilled water for 1h. The filtrates were combined and evaporated under reduced pressure. The residue was dissolved with distilled water up to a final volume of 40mL before administration. The preparation of wine-proceed *G. Radix* solution was the same as the raw one.

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Animals

35 male SD rats (200g±20g), were purchased from Liaoning Chang Sheng Biotechnology Co. Ltd. (Benxi, China), which were kept by adaptive feeding for a week at a temperature of 20-23^{o}C, relative humidity of 50-60 % and well-ventilated environment. All animal experiments were conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals.

Tissue distribution study of gentiopicroside

Drug administration and tissue sampling

35 rats were divided into seven groups (n=5 per group) randomly. Rats were orally administrated with raw and wine-processed *G. Radix* at a single dose of 18.75g/kg. Heart, liver, spleen, lung, kidney, stomach, small intestine and large intestine were collected at 0.5, 1.5 and 4h respectively. Tissue samples were weighed 0.5g rapidly, rinsed with physiological saline to remove blood or contents, blotted on filter paper, then stored in dark place at -20°C before analysis.

Preparation of tissue sample

Each of the weighed tissue samples thawed and then homogenized in ice-cold physiological saline (2mL). Then, a 200μL of tissue homogenate (homogenate time 10 s/time, 3-5 times, ice water bath) was taken and mixed with 50μL internal standard. After protein was precipitated with 600μL of methanol in 1.5mL centrifugal tube by vortexing for 2min, the samples were centrifuged at 8910 ×g for 5 min. The upper clear solution was sucked up and moved to another 1.5mL centrifugal tube, then dried under the nitrogen flow. The rest was dissolved with methanol to 100μL, mixed by vortexing for 2 min and centrifuged at 8910 ×g for 5 min. The upper clear solution was sucked up and moved to a 1.5mL centrifugal tube, 20 μL was injected into HPLC system for analysis.

Method validation

Specificity

The specificity of the method was demonstrated by comparing chromatograms of blank tissue homogenates, tissue homogenate spiked with the analytes and internal standard and tissue homogenate after an oral dose.

Calibration curve

The linearity of the method was assessed by plotting calibration curves in tissue homogenates at five concentration levels. The calibration curves were linear over the concentration range of 0.5-500μg/mL in tissue homogenate of gentiopicroside.

Recovery

The recoveries of gentiopicroside were determined in low, medium and high level of sample. The data indicates that the bio-sample preparation procedure was satisfied and could achieve the acceptable extraction recovery.

Stability

Short-term stability was evaluated by analyzing samples kept at room temperature for 24 h. Long-term stability was studied by analyzing samples following a period of 30-day of storage at -20°C. Freeze-thaw cycles stability was studied by analyzing samples which experienced three cyclic process of freezing and thawing.

Precision

Intraday precision was evaluated by analysis of the six samples with three determination per concentration at the same day. Interday precision was determined by assaying the standard solution of the analysis over 3 consecutive days.

Data analyses

HPLC analysis procedure was applied to analyze tissue distribution of gentiopicroside.

STATISTICAL ANALYSIS

Statistical package for social sciences (SPSS ver 17) was used for analyzing the data. T-test and F-test were applied. *P<0.05 and **P<0.01 was considered as significant when compared against control group.

RESULTS

Method validation

Specificity

Fig. 1 showed that no interference peaks from endogenous components were detected.

Linearity of calibration curve

The calibration curves were linear over the concentration range of 0.5-500μg/mL in tissue homogenate of gentiopicroside. The correlation coefficient values of the calibration curve were over 0.9900. Typical linear regression equations and correlation coefficients in each tissue are listed in table 1.

Recovery / accuracy / precision

The extraction recoveries of gentiopicroside ranged from 79.65±1.87% to 94.40±2.54% in tissue samples. Accuracies were assessed by analyzing six quality control samples. Accuracies of gentiopicroside in tissues ranged from 95.66±1.51% to 106.14±3.78%. The RSD of precisions data for gentiopicroside did not exceed 5.44%. The data are listed in table 2.

Stability

Stability analysis showed no significant sample loss over 24 hours at room temperature, three freeze-thaw cycles and 30 days storage condition. The RSD of three conditions was within 5.23%. The data are listed in table 3.

Tissue distribution study

The tissue concentrations of gentiopicroside determined at 0.5, 1.5 and 4 h after oral administration of raw and wine-

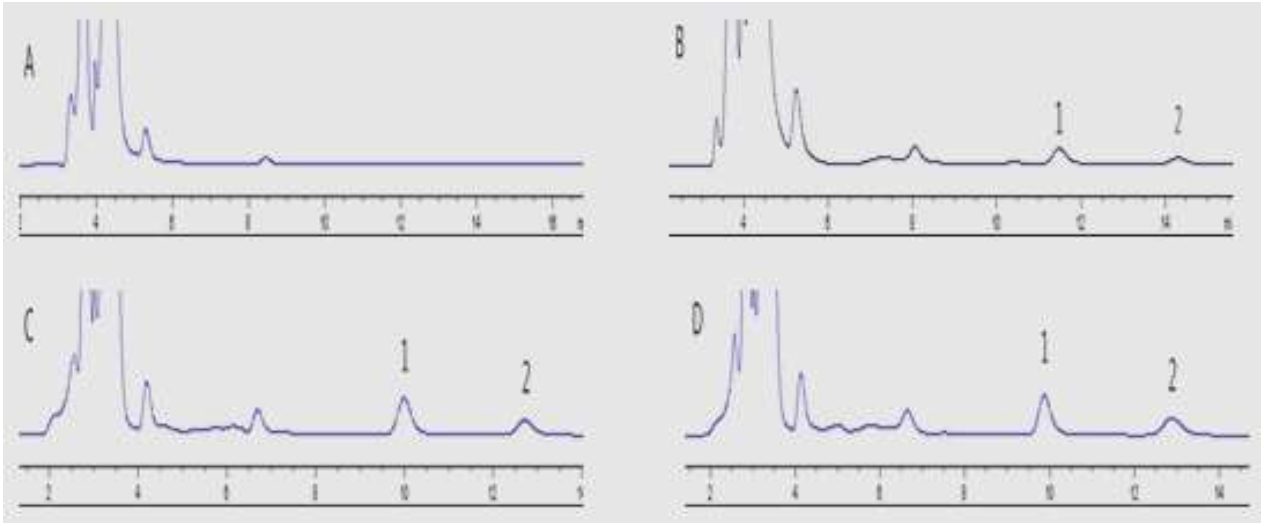

Fig. 1: (A) Chromatograms of blank tissue homogenate. (B) Blank tissue homogenate with gentiopicroside 50μL and Internal standard 50μL. (C) Liver sample (1.5h) after oral administration of raw *G. Radix* 18.75 g/kg. (D) Liver sample (1.5h) after oral administration of wine-processed *G. Radix* 18.75g/kg.(1.caffeine 2.gentiopicroside)

Table 1: The linear regression analysis of gentiopicroside in rat tissue (*n*=6)

Tissues	Standard curves	R	Linear range (μg/mL)
Liver	Y=0.0486X+0.0118	0.999 4	0.5-62.5
Heart	Y=0.0478X+0.0416	0.999 2	0.5-62.5
Spleen	Y=0.0485X+0.0204	0.999 1	0.5-62.5
Lung	Y=0.0479X+0.0152	0.999 6	0.5-62.5
Kidney	Y=0.0488X+0.0146	0.999 4	0.5-62.5
Stomach	Y=0.0419X-0.0424	0.999 6	6.25-500
Small intestine	Y=0.0429X-0.0356	0.999 5	6.25-500
Large intestine	Y=0.0442X+0.1305	0.999 4	2.5-250

processed *G. Radix* at a dose of 18.75g/kg are shown in table 4.

DISCUSSION

The preparation methods of bio-sample include solid phase extraction, liquid-liquid extraction and precipitating protein (methanol, acetonitrile, etc.). Preparing rats tissues with solid phase extraction was not only costly but also complicated. The method of liquid-liquid extraction was time-consuming and laborious (Zhang *et al.,* 2016). In this experiment, the protein precipitation method was used to prepare the bio-samples and the recovery rate of methanol precipitation was higher than acetonitrile precipitation. Therefore, methanol was used to precipitate protein in tissues.

After oral administration of raw and wine-processed *G. Radix*, the content of gentiopicroside in stomach and small intestine tissues were higher than the other tissues because of oral administration. After 1.5h of oral administration, except gastrointestinal tissue, gentiopicroside in the other tissues reached maximum concentration. After 4 h of oral administration, compared with the rats which were treated with raw *G. Radix*, the content of gentiopicroside in the wine-processed one were mostly higher, which indicated that the wine-processed *G. Radix* can stay longer and work better in rats.

Processing can impact on the property of TCM and change their working tendency further (Zhou *et al.,* 2013). After being processed with wine, the property of TCM can be upward. The proportion of gentiopicroside in heart and lung was 0.67 % when the rats were orally administrated with the raw *G. Radix* 0.5h later, while the proportion in wine-processed one was 1.36%. The proportion of gentiopicroside in heart and lung was 1.54% when the rats were orally administrated with the raw *G. Radix* 1.5h later, while the proportion in wine-processed one was 2.83 %. The proportion of gentiopicroside in heart and lung was 3.47% when the rats were orally administrated with the raw *G. Radix* 4h later, while the proportion in wine-processed one was 3.82%. By the theory of traditional Chinese medicine, both heart and lung tissues belong to the upper-JIAO. The result of tissue distribution indicated that distribution of gentiopicroside in upper-JIAO increased when *G. Radix* was processed

Table 2: The recovery/ accuracy /precision of gentiopicroside in rat tissue (*n*=6)

Tissues	Concentration (μg/mL)	Recovery (%)	Accuracy (%)	Intraday precision (RSD%)	Inter-day precision (RSD%)
Liver	100	82.33±1.32	103.26±2.76	2.67	3.46
	25	84.67±2.41	98.36±3.05	3.16	2.76
	6.25	90.45±1.67	96.48±2.79	2.89	4.13
Heart	100	83.56±1.42	104.21±2.88	2.76	2.38
	25	86.48±2.64	97.23±3.42	3.52	2.94
	6.25	91.24±1.62	98.42±2.10	2.13	1.78
Spleen	100	81.46±1.68	95.66±1.51	1.58	3.52
	25	84.33±2.67	101.25±2.47	2.44	5.44
	6.25	90.35±1.09	97.45±2.60	2.67	2.09
Lung	100	81.92±1.72	96.42±2.33	2.42	4.22
	25	83.57±2.68	97.26±3.09	3.18	3.80
	6.25	88.49±2.35	98.36±2.73	2.78	2.35
Kidney	100	79.65±1.87	102.16±2.41	2.36	4.12
	25	82.47±2.54	97.25±2.96	3.04	2.67
	6.25	89.43±2.84	96.28±1.82	1.89	2.73
Stomach	750	89.32±1.89	102.28±3.10	3.03	4.33
	125	91.46±2.12	98.86±3.04	3.08	2.68
	25	93.16±1.28	106.14±3.78	3.56	3.15
Small intestine	750	89.52±2.24	98.42±2.15	2.18	2.67
	125	89.27±1.47	98.53±3.19	3.24	3.52
	25	90.12±1.34	104.18±3.78	3.63	2.84
Large intestine	400	94.40±2.54	97.93±3.20	3.27	3.17
	125	89.36±1.24	97.26±3.15	3.24	2.46
	50	90.32±1.34	98.25±2.28	2.46	3.67

Table 3: The stability of gentiopicroside in liver tissue of rats (*n*=6)

Concentration (μg/mL)	Short-term (RSD%)	Long-term (RSD%)	freeze-thaw cycles (RSD%)
100	3.84	2.24	2.64
25	4.22	3.16	5.23
6.25	3.68	3.86	4.68

Table 4: The tissue concentrations of gentiopicroside after oral administration raw and processed *G. Radix* (*n*=5)

Tissues (μg/g)	0.5 h		1.5 h		4 h	
	raw	processed	raw	processed	raw	processed
Liver	6.44±0.53	7.60±1.94	6.82±1.50	8.42±2.60	2.50±0.44	6.26±1.39*
Heart	2.32±0.72	2.22±0.36	3.64±0.98	5.04±1.06	2.20±0.80	4.00±1.18
Spleen	2.70±0.79	3.20±0.59	10.52±2.19	6.72±1.73	6.66±1.43	5.20±1.60
Lung	3.44±0.86	5.80±1.73	6.40±0.26	15.30±3.84	5.12±1.45	11.80±2.79
Kidney	8.82±2.61	12.32±1.14	21.42±4.53	18.62±3.03	13.68±1.29	12.74±1.73
Stomach	504.90±54.12	432.32±45.42	272.76±37.77	213.78±25.51	96.78±20.63	166.56±21.89
Small intestine	262.36±29.09	94.08±14.99*	219.48±22.15	317.2±6.87	74.06±5.68	143.98±24.26*
Large intestine	75.00±19.99	32.20±12.39*	112.70±17.96	133.4±15.14	9.46±4.02	63.06±16.12**

with wine, which can preliminarily explain the traditional theory of "Processing with wine can make the TCM property upward".

Previous researches have found that *G. Radix* possesses the ability of liver protection (Huang *et al.,* 2016). Compared with the rats administrated raw *G. Radix,* wine-processed one can increase the contents of gentiopicroside in the liver tissue at three time points, such as increasing 18.01% at 0.5h, 23.46% at 1.5h and 150.4% at 4h, which indicated that distribution of gentiopicroside in liver tissue increased when *G. Radix* was processed with wine. This might be the reason that the wine-processed *G. Radix* can enhance the ability of liver protection.

CONCLUSION

The gentiopicroside in raw and wine-processed *G. Radix* was distributed in all tissues and the concentration of gentiopicroside was the highest in the stomach and small intestine. The relative concentration of gentiopicroside in wine-processed *G. Radix* was higher in the upper-JIAO

and liver tissue than raw *G. Radix*, which can explain the processing principle of *G. Radix* from the perspective of tissue distribution.

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