Simultaneous Qualitative and Quantitative Analysis of Commercial Bistorta Rhizome and Its Differentiation from Closely Related Herbs Using TLC and HPLC-DAD Fingerprinting

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A qualitative and quantitative analysis method was established to improve quality assessment standards for Rhizoma Polygoni Bistortae (*Polygonum bistorta* L.) and differentiate commercial bistorta rhizome from closely related herbs by TLC and HPLC-DAD fingerprinting. Three compounds including phenolic acid and flavane were identified by comparison with standard compounds and quantified simultaneously by HPLC-DAD simultaneously. A comprehensive validation of the method that included sensitivity, linearity, repeatability and recovery was conducted. *Paris polyphylla* Sm., a herb often mixed with *Polygonum bistorta* L. in China due to their same popular name "Caoheche" in history, was successfully distinguished by thin-layer chromatography (TLC) fingerprinting of the petroleum-soluble fraction. *Polygonum paleaceum* Wall., another herb often mixed with *Polygonum bistorta* L. due to their similar external appearances, was distinguished by HPLC fingerprinting.

Key words *Polygonum bistorta*; *Polygonum paleaceum*; *Paris polyphylla*; TLC; HPLC; quality assessment

Rhizoma Polygoni Bistortae (quanshen, QS), the dried rhizome of *Polygonum bistorta* L. (Polygonaceae), is a widely used traditional Chinese medicinal material in China and is listed in the Chinese Pharmacopoeia (2005 edition). It has been used to treat dysentery with bloody stools, diarrhea in acute gastroenteritis, and venomous snake bites, among others in traditional Chinese medicine (TCM).[1)] Chemical studies have shown that tannin, flavonoids, phenolic acids and triterpenoids are the major chemical constituents.[2—4)] Modern pharmacological and clinical studies have indicated that the extract of *Polygonum bistorta* L. has antibacterial, anti-inflammatory and antitumor activities.[4—6)]

Paris polyphylla Sm. (chonglou, CL), a member of the family Liliaceae, is a herb often mixed with QS, due to their same popular name "Caoheche" in many books on TCM. However, its chemical constituents and target conditions and diseases are entirely different from QS. CL is used for the treatment of boils, carbuncles, sore throat, traumatic pain, and convulsion.[1)] *Polygonum paleaceum* Wall. (caoxuejie, CXJ), a member of the family Polygonaceae, is an another herb often mistaken for QS due to their similar morphological appearances as well as chemical constituents. In traditional Chinese medicine, CXJ is used for the treatment of chronic gastritis, duodenal ulcers, dysentery, wound, pain, inflammation, hemorrhage and irregular menstruation.[7)] CL and CXJ are often mistaken for QS in China.[8)] This mistake in identification has resulted in the abuse of the plants of different families and genera, and has even resulted in harm to patient health. Therefore, it is important and necessary to distinguish QS from its closely related herbs so that traditional Chinese medicine can be used safely and effectively in clinical situations.

It is well accepted that herbal medicines can be distinguished from each other by comparing their chemical constituents. Fingerprinting is a method that shows chemical information of TCM with chromatograms, spectrograms and other graphs.[9)] In this paper, TLC-fingerprinting profiles were generated with the aim of differentiating QS and CL. Representative HPLC-fingerprinting profiles were generated with the aim of differentiating QS and CXJ based on their identities and relative amounts of chemical constituents.

Experimental

Herbal Materials Samples of QS (No. 1—14) , CXJ (No. 15—18) and CL (No. 19—20) were obtained from different regions of China. The sources of the plant materials are listed in Table 1. These samples were authenticated by Prof. Yun-Zhen Guo, Department of Pharmacognosy, Shenyang Pharmaceutical University (Shen Yang, China).

Reference Standards The reference compounds of gallic acid, chlorogenic acid and catechin (purity >98%) were purchased from the National Institute for the Control of Pharmaceutical Biological Products (Beijing, China). 24(*E*)-ethylidenecycloartanone, epifridelanol and β-sitosterol (purity >95%) were isolated and identified in our laboratory, and purities were confirmed by HPLC. The ^{1}H- and ^{13}C-NMR data of epifridelanol[10)] and 24(*E*)-ethylidenecycloartanone[4)] were compared with references and corresponded to the data of epifridelanol and 24(*E*)-ethylidenecycloartanone.

Solvents and Chemicals Acetonitrile and methanol of HPLC grade were purchased from Concord Technology Co., Ltd. (Tianjin, China). The deionized water for HPLC analysis was purchased from Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China). Acetic acid and other solvents of analytical grade were purchased from Shandong Yuwang Group Co., Ltd. (Shandong, China).

TLC Conditions TLC silica gel plates (10 cm×20 cm) were prepared in our laboratory. The plates were dried for 30 min at 105 °C before use. The plates were pre-equilibrated for 30 min with the mobile phase. Petroleum ether–acetone (10 : 1) was chosen as the solvent system for TLC separation. After developing over a path of 10 cm, the plates were air-dried, sprayed with 10% H_2SO_4/EtOH, heated at 110 °C for 10 min, and then imaged immediately under visible light and UV light (365 nm).

HPLC Conditions for Fingerprinting The HPLC system consisted of a Shimadzu LC-10ATVP chromatograph and a SPD-M10AVP detector. The LC separation was performed on a Kromasil-C_{18} column (5 μm, 250×4.6 mm) protected by a guard C_{18} column (5 μm, 4×40 mm) from Tianjin Pharmacokinetic Technology Co., Ltd. (Tianjin, China) at room temperature. Data were acquired and processed using Shimadzu Class-vp 6.12 software. Solvents that constituted the mobile phase were A (methanol with 1% acetic acid) and B (1% aqueous acetic acid). The elution conditions applied were: linear gradient 0—10 min: 0→5% A; 10—20 min: isocratic 5% A; 20—40 min: linear gradient 5%→30% A; 40—50 min: linear gradient 30%→35% A; 50—60 min: linear gradient 35%→100% A. 60—65 min: isocratic 100% A; and finally, the reconditioning step of the column was 100% B isocratic for 15 min. The flow-rate was 0.8 ml/min and the injection volume was 20 μl. The detection wavelength for fingerprint profiles was set

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at 280 nm.

HPLC Conditions for Quantitation For gallic acid and catechin, the detection wavelength was 272 nm and for chlorogenic acid, 327 nm was selected. The elution conditions, the flow-rate and the injection volume were the same as the conditions for HPLC fingerprinting.

Sample Preparation for TLC Identification The powdered sample (2.0 g, passed through a 500 μm mesh) was extracted with 25 ml of petroleum ether (bp 60—90 °C) for 4 h by soaking, then 20 min by sonication. After filtration, the filtrate was quickly dried in a water bath. The residue was then dissolved in 1 ml of chloroform as the TLC test solution.

Sample Preparation for HPLC Fingerprinting The powdered sample (0.25 g, passed through a 355 μm mesh) was extracted with 25 ml of 30% methanol for 40 min by soaking, then 20 min by sonication. The supernatant was filtered through a 0.45 μm filter into an HPLC amber sample vial for HPLC fingerprinting analysis.

Identification of Peaks in HPLC Fingerprints The peaks in HPLC fingerprinting were identified by comparing the retention times of gallic acid (peak 1), catechin (peak 4) and chlorogenic acid (peak 5) in chromatograms of the extracts with those of the authentic reference standards.

Preparation of Standard Solutions for HPLC Quantitation Gallic acid (1.18 mg) chlorogenic acid (3.02 mg) and catechin (2.25 mg) were accurately weighed, placed into 10 ml volumetric flasks, diluted with methanol to volume, and mixed to make standard solutions.

Results and Discussion

Paris polyphylla (CL) and *Polygonum paleaceum* (CXJ) are commonly mixed with *Polygonum bistorta* (QS) at local markets and in clinical situations. Therefore, it is important and necessary to distinguish them from each other. We established a TLC fingerprinting method to study the difference between QS and CL. The chromatographic conditions, especially the developing solvents (type of solvents and ratio), were carefully optimized before 20 batches of samples from different areas were analyzed. The results observed under UV light showed a good separation for the petroleum-soluble fraction (Figs. 1, 2). It showed that 24(*E*)-ethylidenecycloartanone, epifridelanol and β-sitosterolol were not found in CL, so we can distinguish QS from CL by TLC. It was reported that the 3 compounds were isolated from the rhizomes of *Polygonum bistorta*,[3] which has also been confirmed by our investigations. 24(*E*)-ethylidenecycloartanone is a new compound, and epifridelanol was isolated from polygonum for the first time. The contents of the 3 compounds were large in rhizomes of *Polygonum bistorta*, whose reference substances were obtained easily. The 3 compounds may represent non-polar chemical constituents of *Polygonum bistorta*. In addition, the 3 compounds did not exist in rhizomes of *Paris polyphylla* Sm. Therefore, TLC analysis for the 3 compounds, 24(*E*)-ethylidenecycloartanone, epifridelanol and β-sitosterolol, was effectively used to distinguish the two. However, CXJ has non-polar chemical constituents similar to those of QS, which can not be distinguished from each other by TLC fingerprinting when using petroleum-soluble fractions. For this reason, an HPLC chromatographic method was used to distinguish QS from CXJ.

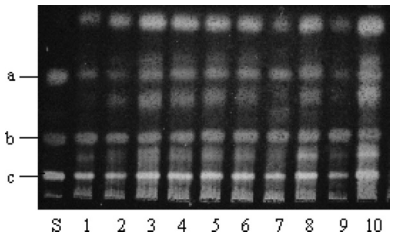

Fig. 1. TLC of Reference Substances and *P. bistorta* L. (UV 365 nm)

S: 24(*E*)-ethylidenecycloartanone (a), epifridelanol (b) and β-sitosterol (c); No. 1—10: samples of *Polygonum bistorta* L.

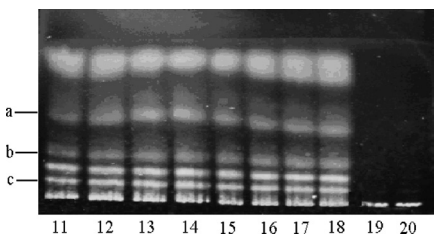

Fig. 2. TLC of *P. bistorta* L., *P. paleaceum* and *Paris polyphylla* (UV 365 nm)

No. 11—14: *P. bistorta*, 15—18: *P. paleaceum*, 19—20 : *Paris polyphlla*.

Optimization of HPLC Extraction Solvent Water, 30, 50, 70% and 100% methanol were selected as extraction solvent in this study. The QS samples were extracted with 25 ml of solvent for 20 min in an ultrasonic bath, and the extraction solution was filtered through a 0.45 μm filter. As a result, the maximum peak area obtained with 30% methanol was larger than those for the other solvents.

Optimization of HPLC Chromatographic Conditions It has been reported that components of phenolic acids can be separated by acetonitrile–water–acetic acid.[11] After comparing acetonitrile–water–acetic acid with methanol–water–acetic acid, we found that the presence of acid in a mobile phase system can improve peak trailing of compounds and change the capacity factor of components. However, the acetonitrile–water–acetic acid system did not improve the separation of peaks. Therefore, a methanol–water–acetic acid mobile phase system was considered for this chromatographic condition.

The concentration of acetic acid in the mobile phase was varied from 0.0 to 1.5%. The calculated capacity factor increases as the concentration of acetic acid increases (Fig. 3). The peaks of gallic acid, 2, 3 and chlorogenic acid exhibited better separation when 1.0% of acetic acid was chosen so 1.0% acetic acid was selected.

Full UV spectra (190—370 nm) of QS were obtained by the DAD-detector. Spectrograms of the constituents determined are shown in Fig. 4. Gallic acid, peak 2, peak 3 and catechin could be resolved with baseline at 272 nm and better resolution of chlorogenic acid could be obtained at 327 nm. Therefore, a monitoring wavelength of 272 nm was used for the quantitative determination of gallic acid and catechin, while 327 nm was used for chlorogenic acid. The fingerprint profiles were recorded at an optimized wavelength of 280 nm.

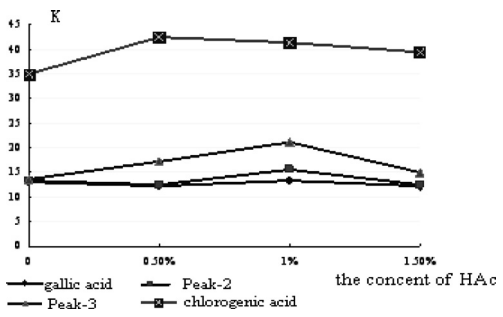

Fig. 3. Effect of Acetic Acid (HAc) on Capacity Factor (K)

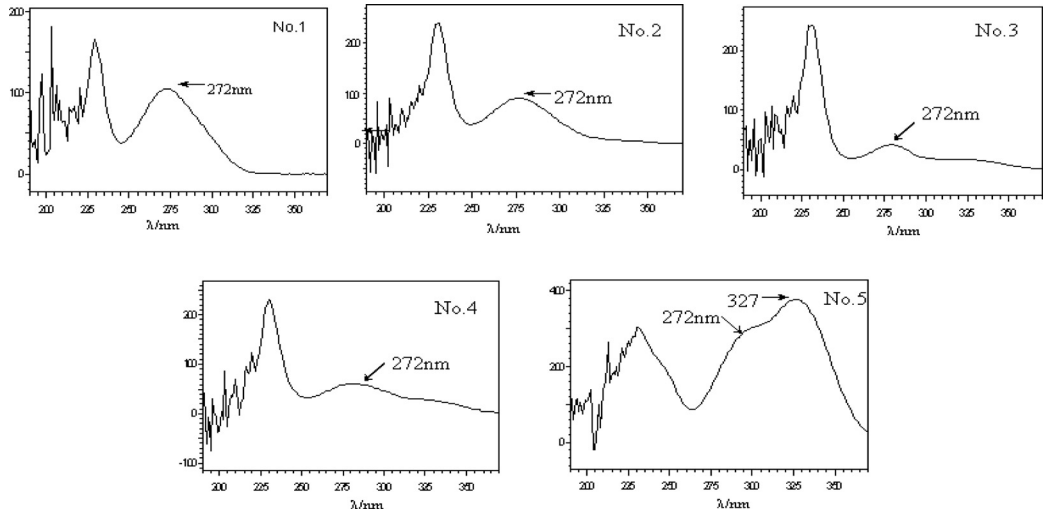

Fig. 4. UV Spectra of the Key Peaks

No. 1. gallic acid; No. 2, 3. unknown; No. 4. catechin; No. 5. chlorogenic acid.

Table 1. Contents of Gallic Acid, Chlorogenic Acid, and Catechin in Samples Used in Study (n=3)

No.	Markets	Species of the toads	Data	Gallic acid (%)	Chlorogenic acid (%)	Catechin (%)
1	Shenyang, Liaoning Prov.	*Polygonum bistorta*	Sep. 2002	0.69	0.80	1.01
2	Qinhuangdao, Hebei Prov.	*Polygonum bistorta*	Feb. 2003	0.46	0.99	0.33
3	Weifang, Shandong Prov.	*Polygonum bistorta*	Feb. 2003	0.62	0.89	1.07
4	Jiaohe, Jilin. Prov.	*Polygonum bistorta*	Dec. 2002	0.51	0.91	0.45
5	Dalian, Liaoning Prov.	*Polygonum bistorta*	Feb. 2003	0.85	0.93	1.70
6	Rugao, Jiangsu Prov.	*Polygonum bistorta*	Feb. 2003	0.24	0.68	0.36
7	Haerbin, Heilongjiang Prov.	*Polygonum bistorta*	Dec. 2002	0.37	0.67	0.68
8	Shenyang, Liaoning Prov.	*Polygonum bistorta*	Feb. 2004	0.88	0.71	1.42
9	Guiyang, Guizhou Prov.	*Polygonum bistorta*	Jul. 2003	0.28	0.69	0.57
10	Wenshui, Shanxi Prov.	*Polygonum bistorta*	Jul. 2003	0.28	1.07	0.73
11	Datong, Shanxi Prov.	*Polygonum bistorta*	Aug. 2003	0.39	0.93	0.44
12	Wuchang, Hubei Prov.	*Polygonum bistorta*	Aug. 2003	0.50	0.69	0.87
13	Bengbu, Anhui Prov.	*Polygonum bistorta*	Aug. 2003	0.57	1.52	0.65
14	Neimenggu Prov.	*Polygonum bistorta*	Jul. 2003	0.29	0.60	0.53
	Average			0.50	0.86	0.77
15	Guiyang, Guizhou Prov.	*P. paleaceum*	Mar. 2004	0.12	0.30	0.20
16	Kunming, Yunnan Prov.	*P. paleaceum*	Mar. 2004	0.03	0.16	0.16
17	Guiyang, Guizhou Prov.	*P. paleaceum*	Mar. 2004	0.06	0.10	0.10
18	Guiyang, Guizhou Prov.	*P. paleaceum*	Mar. 2004	0.07	0.04	0.09
	Average			0.07	0.15	0.14

Validation of the Method and Quantification of Major Constituents in QS Comprehensive validation of the present method was conducted and the results indicated that good linearity with a correlation coefficient of $R^2>0.99$ was achieved for gallic acid, chlorogenic acid and catchin, and the regression lines were $Y=4.3495\times10^6X+3.67\times10^2$, $Y=7.8\times10^4X-4.24\times10^5$ and $Y=1.82\times10^4X-4.7\times10^3$, with linearity range: 14.8—118.4 μg/ml, 30.2—302 μg/ml and 22.5—225μg/ml, respectively. Precision was evaluated and relative standard deviation (RSD) values were within 0.44—1.85%. Repeatability was tested and the RSD values were within 1.46—2.25%. The recovery rates were 100.2% (RSD 1.74%), 99.6% (RSD 2.53%) and 95.8% (RSD 2.14%), respectively.

Analysis of QS and CXJ Samples from Various Markets In the present investigation, we found that commercial bistort rhizomes were commonly mixed with a closely related herb, *Polygonum paleaceum*, which resulted in improper clinical use. In this paper, a method was established to analyze commercial Bistorta Rhizome, improve its quality assessment standard by chromatographic fingerprintings, and quantify the compounds in the crude drug.

The amounts of gallic acid, chlorogenic acid, and catechin (Table 1) were rather large in *Polygonum bistorta*. Flavanes and phenolic acids have anti-bacterial and anti-tumor activities.[5,6] Therefore, we believe the 3 compounds including gallic acid, chlorogenic acid and catechin are the bioactive components of Rhizome Polygoni Bistortae.

The difference can be visualized in terms of peak abundance through the fingerprinting profiles of QS and CXJ (Fig. 5). In 18 batches of QS samples, it was found that commercial bistorta rhizome samples from various markets had different contents. We think different regions, different growing conditions and different collection times may be the main

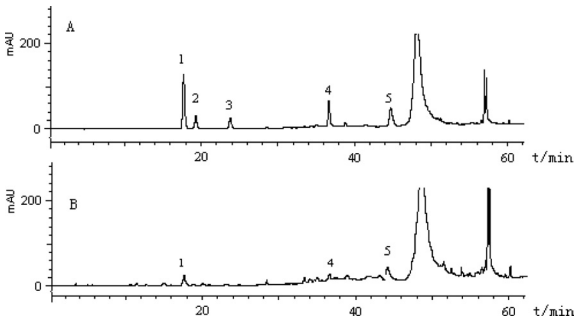

Fig. 5. Chromatograms of *Polygonum bistorta* (A) and *Polygonum paleaceum* (B) at 280 nm

1. gallic acid; No. 2, 3. unknown; 4. catechin; 5. chlorogenic acid.

reasons for the differences in contents. Moreover, the samples have been treated with water to keep clean, and the 3 compounds may be lost in the procedure. By analyzing the contents of marker compounds, the results show that there were peaks 2 and 3 were present in the HPLC chromatogram of *Polygonum bistorta*, while the peaks were not present in *Polygonum paleaceum*, and the contents of gallic acid, chlorogenic acid and catechin in QS were always higher than those in CXJ, which can also be used to distinguish from *Polygonum paleaceum*.

Therefore, it is important and necessary to distinguish rhizomes of *Polygonum paleaceum* so that traditional Chinese medicines can be used safely and effectively in clinical situations.

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