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Research Article
Chromatographic Fingerprint Analysis is Feasible for Comprehensive Quality Control of Taohongsiwu

[1,2]Lan Han, [1]Ou Qiao, [1]Huanru Wu, [1]Shan Wu, [1]Yanyan Zhang, [1]Liang Yao, [1]Kangjing He, [1,2]Weidong Chen and [1,2]Daiyin Peng

[1]School of Pharmacy, Anhui University of Chinese Medicine, 230012 Hefei, China
[2]Anhui Provincial Key Laboratory for Chinese Medicine Research and Development, 230038 Hefei, China

Abstract

Background and Objective: Taohongsiwu decoction, a traditional Chinese medicine formula has been widely developed to treat thrombotic diseases for hundreds of years. It contains variations of multi-components, which pose a serious challenge to its quality control. This study aimed to develop a simple and reliable ultra high performance liquid chromatography coupled with diode array detector method to separate and detect the constituents of the decoction. **Materials and Methods:** A total of 15 compounds was detected and 6 compounds (including ferulic acid, hydroxysafflor yellow a, ligustilide, amygdalin, 5-hydroxymethyl furfuraldehyde and paeoniflorin) were tentatively identified, using high performance liquid chromatography coupled with diode array detector method. **Results:** The four components (Ferulic acid, hydroxysafflor yellow a, ligustilide, amygdalin) showed good regression (R>0.9996) within test ranges and the recovery method ranged from 101.73-106.44%. Ten batches of taohongsiwu decoction were analyzed. The similarity scores of common peaks from these samples ranged from 0.957-1.000, which indicating that samples were highly correlated. **Conclusion:** It is concluded that this extraction method was found to be sensitive and simple, so it was feasible for comprehensive quality control of THSWD.

Key words: Chromatographic fingerprint, quality control, taohongsiwu decoction, traditional Chinese medicine, ultra high liquid chromatography

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Corresponding Authors: Daiyin Peng and Weidong Chen, School of Pharmacy, Anhui University of Chinese Medicine Qianjiang Road, 230012 Hefei, China
Tel/Fax: +86 55168129028/+86 55168129028

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Taohongsiwu decoction (THSWD) is a distinguished traditional Chinese medicine formula, which has been used for the treatment of blood deficiency and blood stasis for ages[1]. It consists of six species of Chinese herbal pieces of: Saudi (Rehmanniae Radix Praeparata), Bai shao (Paeoniae Radix Alba), Danggui (Angelicae Sinensis Radix), Chuanxiong (Chuanxiong Rhizoma), Taoren (Persicae Semen) and Honghua (Carthami Flos). In previous several pharmacological studies[1,2] established that it possessed anti-thrombosis effect, anti-platelet activation, THSWD also has an effect on postpartum blood stasis, since it can nourish blood and promote blood circulation. It is generally recognized that the decoction involves many bioactive constituents, such as amygdalin, ferulic acid, ligustilide and hydroxysafflor yellow A. Previous studies[3-9] revealed that the four bioactive constituents exhibited neuroprotective activity, angiogenesis, protective effect for endothelial cells. In this study, the four bioactive constituents were selected to quantify. Due to the merits of obvious therapeutic effects and wide effect, as the development of new drugs require the safe, effective and controllable, the quality control of THSWD is very important.

Up to date, only a few analytical techniques have been reported to qualitative or quantitative analysis of some active components in THSWD, such as High Performance Liquid Chromatography (HPLC) and ultra performance liquid chromatography quatrupole-time of flight mass spectrometry (UPLC-QTOF-MS)[10]. The methods used in previous reports[11,12] only focused on quantitative analysis of some active components, but ignoring the entirety and complexity of the THSWD, these can not be used as quality control standards. Therefore, a simple, reliable and fast method is needed to quantify the compounds in THSWD, which is helpful for controlling the quality, tentatively identified some active components of this famous decoction and revealing the superiority of combined use of single herbs.

Now-a-days analysis of the chemical composition of traditional chinese compound medicine (TCM) is moving in the integrative and holistic direction[13,14]. The quality of judgment by determining, selecting one or two markers, ignoring the entirety and complexity of the Chinese herbal medicine was no longer appropriate for TCM research. It is well known that the one Chinese herbal medicine may contain hundreds of chemical components, which may hinder further understanding of its bioactive constituents[15]. Moreover, the therapeutic effect is also based on the synergistic effect of multiple ingredients according to the Chinese medicine theory, which makes TCM different from Western medicines[16]. The fingerprint technique is considered as an effective method to control TCM quality because it describes all the characteristics of TCM[17], it not only determines the characteristic patterns of each plant type but also reveals the inherent relationships between multiple compounds[18]. Therefore, due to the advantages of fingerprint, it has been widely applied by many organizations including United States Pharmacopoeia, European Pharmacopoeia and Chinese Pharmacopoeia[19].

This study aimed to develop a method for quality control of THSWD. In this study, a simple, accurate and practical Ultra High Performance Liquid Chromatography (UHPLC) method could set up characteristic fingerprint of single component, lack of one drug in formula and compound recipe and to quantify the active constituents amygdalin, ferulic acid, ligustilide and hydroxysafflor yellow A. The fingerprint provided a basis for the quality control of the decoction and chemical information to further study its potential therapeutic material basis.

MATERIALS AND METHODS

Materials: All crude materials were purchased from Bozhou Yonggang Pieces Factory Co. Ltd. (Anhui, China). Amygdalin, ferulic acid, ligustilide, hydroxysafflor yellow A, ligustrazine, paeoniflorin, 5-hydroxymethyl furfuraldehyde, acteoside (batch number: 140127, 140508, 140815, 150528, 140209, 140401, 150612, 151121) were provided by Weikeqi Biotech Corporation (Sichuan), all eight reference compounds used in the analysis had purities >98%.

Methanol (HPLC grade) was purchased from Merck-Corporation (Darmstadt, Germany) and acetic acid (Chromatographic grade) was purchased from Qiangsheng chemical limited by Share Ltd. (Jiangsu, China) ultrapure water was purified by a Milli-Q system (Millipore, Bedford, USA). The other organic reagents were analytical grade.

Preparation of sample solution: Six materials (17 g, at a weight ratio of 4:3:3:2:3:2) were immersed in a 10 fold volume of 75% ethanol (w/v = 1:10) for 30 min, then boiled for 2 h and filtrate was collected. The residue was refluxed again for 2 h, with 8 fold 75% ethanol (w/v = 1:8). Then the decoction obtained was mixed and concentrated, then transferred to 50 mL volumetric flask and dissolved in ultrapure water to a final volume of 50 mL to obtain reserve concentration of 340 mg mL^{-1}. The final solution was filtered through 0.22 μm membrane filters before use. An aliquot of 2 μL of each sample solution was injected into the UHPLC system for analysis.

Preparation of standard solution: Eight compounds were separately weighted and dissolved in methanol as stock standard solution. Then the appropriate volumes of each stack solution were mixed together to produced a solution containing 0.454 μg μL^{-1} ferulic acid, 1.244 μg μL^{-1} hydroxysafflor yellow A, 0.480 μg μL^{-1} acteoside, 0.528 μg μL^{-1} ligustrazine, 1.006 μg μL^{-1} 5-hydroxymethyl furfuraldehyde, 0.801 μg μL^{-1} ligustilide which, 0.660 μg μL^{-1} amygdalin and 0.398 μg μL^{-1} paeoniflorin, which was used as the reference solution.

Instrument and chromatographic condition: The 1290 UHPLC system (Acquity, USA) comprised a four-element high pressure gradient pump, a vento, an auto-injector, a column temperature controller and a diode array detector. A ACQUITY CSH C_{18} column (2.1×100 mm, 1.7 μm) was used in the UHPLC system.

The detection wavelength used was as follows: 284 nm from 0-4.5 min, 210 nm from 4.5-9.0 min, 230 nm from 9.0-13.0 min, 295 nm from 13.0-18.0 min, 350 nm from 18.0-30.0 nm and the column temperature was set at 30. The mobile phase were A (0.05% acetic acid solution 400 mL+ammonium acetate 154.4 mg) and B (methanol). The flow rate was set at 0.2 mL min^{-1}. The injection volume was 2 μL. The gradient system used was as follows: 90-80% A from 0-3 min, 80-75% A from 3-6 min, 75-70% A from 6-17 min, 70-60% A from 17-19 min, 60-40% A from 19-22 min, 40-20% A from 22-24 min, 20-40% A from 24-28 min and 40-90% A from 28-30 min.

Data analysis: Date analysis for chromatographic fingerprint was performed by use of the professional analysis software 'Similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine (Version 2012.1). Using this software, the correlative coefficient for samples were calculated and the similarities of different Chinese medicine fingerprint were compared with the average chromatogram among the samples.

RESULTS

Optimization of UHPLC conditions: Different UHPLC parameters were examined and compared, to obtain as much chemical information as possible and to choose the best separation conditions in chromatograms. It was found that methanol in-0.05% acetic acid solution 400 mL+ammonium acetate 154.4 mg as mobile phase provided a better resolution and separation of the THSWD.

Table 1: Precision, reproducibility, stability and recovery of the four bioactive constituents (n = 6)

Compounds	Precision RSD (%)	Reproducibility RSD (%)	Stability RSD (%)	Recovery (%)
Ferulic acid	0.0019	0.0249	0.0271	106.44
Amygdalin	0.0049	0.0303	0.0175	101.73
Ligustilide	0.0007	0.0272	0.0317	104.37
HSYA	0.0011	0.0237	0.0266	102.43

RSD: Relative standard deviation

Table 2: Regression data for the four bioactive constitutes including regression equation, R and linear range

Compounds	Regression equation	R	Linear range (μg mL^{-1})
Ferulic acid	Y = 39.641X+22.19	0.9996	3.16-60
Amygdalin	Y = 10.092X+0.4119	0.9999	11.87-150
Ligustilide	Y = 15.333X+3.3023	0.9999	14.24-180
HSYA	Y = 21.653X+6.6717	0.9999	18.98-240

Precision, reproducibility, stability, recovery and linearity of four bioactive components: The quantitative method was assessed by precision, repeatability, stability, reproducibility and linearity. Ten batches of THSWD were determined by UHPLC under the above-mentioned optimum conditions and the assay results are listed in Table 1. Recoveries were also determined to evaluate the precision and accuracy of the method.

Standard solutions containing four compounds were diluted to appropriate concentrations for calibration curve construction. The solutions at seven different concentrations and the calibration curves were established by plotting the peak area (y) versus the concentration (x) of each component. The detailed information regarding the calibration curves and linear ranges of the four compounds are summarized in Table 2. The results indicate that this method is accurate, sensitive, reliable and reproducible and it is a useful method for quantitative analysis of the four bioactive components in THSWD.

Fingerprint of THSWD: With UHPLC method, 10 batches of samples were analyzed in the optimum conditions. The average chromatogram from the 10 batches was regarded as the standardized characteristic fingerprint of THSWD. Peaks existed in all chromatograms of these samples were assigned as "Common peaks", indicating the sameness among various samples. The chromatograms of THSWD from the samples consisted of 15 common peaks within 30 min, shown in (Fig. 1). Among these components, 5-hydroxymethyl furfuraldehyde (retention time 3.38 min) indicated a high degree of separation and stable content, therefore it was chosen as the reference substance. Samples from 10 batches share similar chromatographic patterns and are relatively

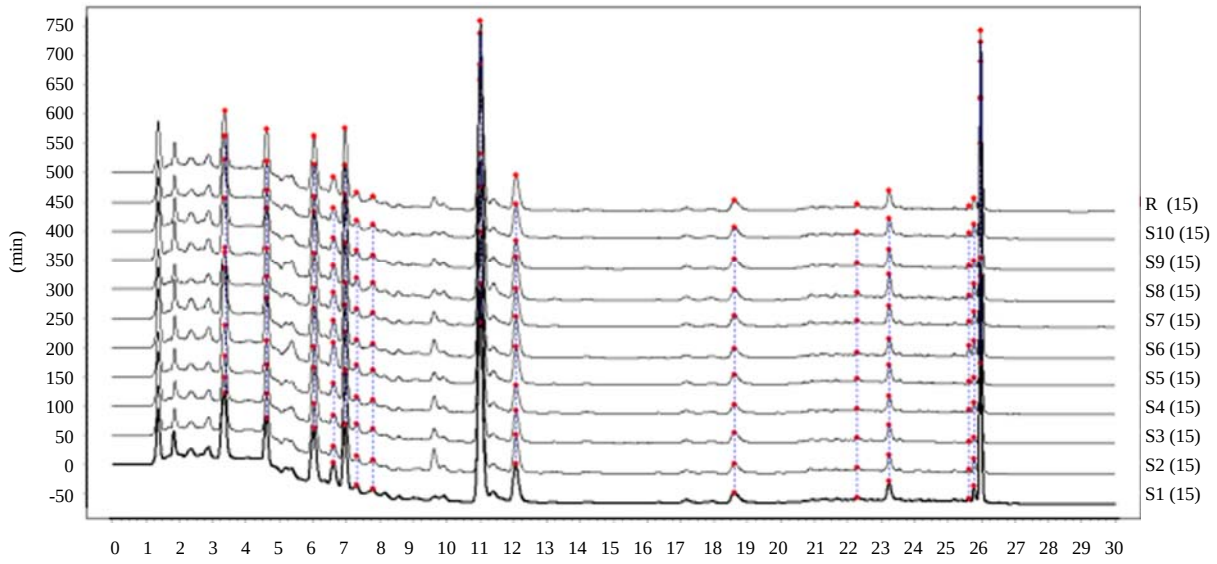

Fig. 1: UHPLC results

Chromatograms of THSWD from the samples consisted of 15 common peaks within 30 min

Table 3: Analysis of similarities among 10 compared samples

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Comparison
S1	1	0.98	0.97	0.99	0.98	0.99	0.99	0.99	0.99	1	0.996
S2	0.98	1	0.97	0.98	0.96	0.99	1	0.98	0.97	0.99	0.989
S3	0.97	0.97	1	0.99	0.99	0.94	0.96	0.98	0.98	0.97	0.983
S4	0.99	0.98	0.99	1	1	0.97	0.98	0.99	1	0.99	0.996
S5	0.98	0.96	0.99	1	1	0.95	0.96	0.99	0.99	0.98	0.989
S6	0.99	0.99	0.94	0.97	0.95	1	1	0.97	0.96	0.99	0.984
S7	0.99	1	0.96	0.98	0.96	1	1	0.99	0.97	1	0.992
S8	0.99	0.98	0.98	0.99	0.99	0.97	0.99	1	0.99	0.99	0.995
S9	0.99	0.97	0.98	1	0.99	0.96	0.97	0.99	1	0.98	0.991
S10	1	0.99	0.97	0.99	0.98	0.99	1	0.99	0.98	1	0.998
Comparison	1	0.99	0.98	1	0.99	0.98	0.99	1	0.99	1	1

Table 4: Relative Standard Deviation (RSD) valves of repeatability, stability, inter-day and intra-day precision

No. of peaks	Retention time (min)	Stability RSD (%)	Repeatability RSD (%)	Inter-day precision RSD (%)	Intra-day precision RSD (%)
1	3.38	0	0	0	0
2	4.65	0.34	0.87	7.09	0.72
3	6.13	0.29	0.55	4.91	0.38
4	6.69	0.24	0.2	1.95	0.48
5	7.01	0.22	0.27	2.72	0.2
6	7.37	0.22	0.29	1.14	0.18
7	7.87	0.28	0.58	1.67	0.28
8	11.1	0.3	0.2	6.42	0.52
9	12.14	0.49	0.52	6.3	0.38
10	18.95	1	0.58	4.28	0.97
11	22.34	1.9	0.49	0.77	0.67
12	23.28	2.44	0.43	0.85	0.12
13	25.63	2.43	0.01	0.23	0.01
14	25.79	2.43	0.04	0.61	0.29
15	25.99	2.44	0.81	3.7	0.5

RSD: Relative standard deviation

consistent (Table 3). The fingerprint method was assessed by repeatability, stability, inter-day and intra-day precision (Table 4). Low RSD valves demonstrate that good precision of instrument. So, the peak of the all components made up the fingerprint of THSWD besides the 15 common peaks (Fig. 2).

Correlation between THSWD and its raw herbal medicines: Comparing the relative retention time of 15 peaks in THSWD fingerprint with that one of the herbals and lack of one of the herbals (Table 5). Peak No. 1 (5-hydroxymethyl furfuraldehyde) was chosen as the reference component, which exists in the Rehmanniae Radix Praeparata.

DISCUSSION

In this study, the similarity of 10 batches of THSWD was more than 0.95 that shows the samples belong to the same

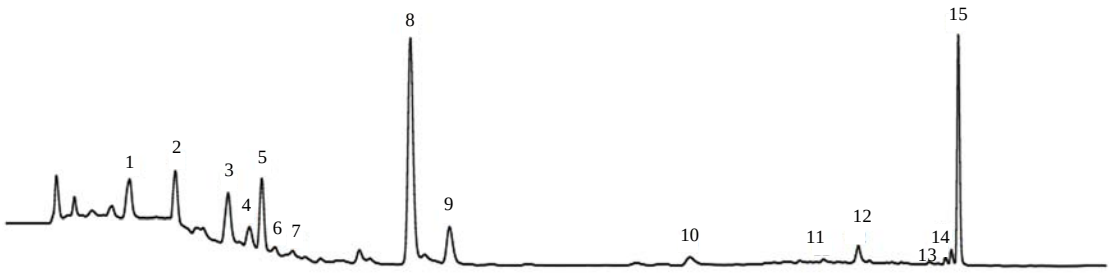

Fig. 2: Fingerprint results

Low RSD valves demonstrate good precision of instrument. So the peak of the all components made up the fingerprint of THSWD besides the 15 common peaks

Table 5: Correspondence of peaks between THSW and raw herbs and peak numbers based on the Fig. 2

Medicinal herbs	Peak No.														
THSWD	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Saudi	1														
Danggui									9					14	15
Baishao			3	4				8							
Chuanxiong									9						15
Taoren				4	5										
Honghua		2	3			6	7			10	11	12	13		

family and select same using part, which from the same origin place were consistent. According to UV data with reference standards and retention times six peaks in the chromatograms were successfully confirmed as corresponding to amygdalin, ferulic acid, ligustilide and hydroxysafflor yellow A. The chromatographs of six raw herbal medicines were also established by UHPLC for quality analysis and the major common peaks in THSWD fingerprint was found in their raw herbal fingerprints, showing the co-relation between them.

Single wavelength detection method can be applied to single herb analysis since the major marker constituents are often concentrated with one or a few chemical constituents and typically would have similar UV absorption wavelength. However, for traditional Chinese formulas that are often consisted of as many as a dozen herbs single wavelength quantitative analysis may not be sufficient. Previous studies on fingerprint methods for traditional Chinese formulas have mainly used only single wavelength detection to detect and/or quantify only a few compounds acting as bio-markers in the sample[20]. Methods incorporating dual-wavelength and multi-wavelength detection could be more suitable for the quality control of traditional Chinese formulas[21,22]. Based on the maximum absorption and full-scan experiment of the marker components in the UV spectra of the three-dimensional chromatograms obtained by Diode Array Detection (DAD), the detection wavelengths was 210, 230, 284, 295, 316, 334, 350 and 403 nm. According to the retention time and absorption profiles of bioactivity components, five wavelengths, 210, 230, 284, 295 and 350 nm were used for detection.

According to the Chinese Pharmacopoeia[23], ferulic acid is used as the marker substance to evaluate the quality of Angelicae Sinensis Radix and Chuanxiong Rhizoma. Amygdalin and HSYA are used as marker compounds for the quality control of Persicae Semen and Carthami Flos respectively. And ligustilide is also as the main volatile oil of Angelicae Sinensis Radix and Chuanxiong Rhizoma. So the amygdalin, ferulic acid, ligustilide and hydroxysafflor yellow A in THSWD were determined at the above-mentioned optimized conditions. ferulic acid, is an important pharmaceutically active agent in the treatment of leukopenia and in providing protection against cardiovascular and cerebrovascular disease[24]. It has also been shown to possess antiatherogenic, antidepressant and antioxidant properties[25,26].

Amygdalin, a quality marker of TR, has been reported to treat asthma, aplastic anemia and tumors in oriental medicine[27]. Amygdalin is also commonly used as an expectorant and supplementary anti-cancer drug[28]. Lee and Moon[29] suggested a potential application of amygdalin as a chemopreventive agent to prevent or alleviate progression of breast cancer, especially triple-negative breast cancer. Ligustilide has been used clinically to treat cardiovascular and cerebrovascular diseases and primary dysmenorrhea. Hydroxysafflor yellow

A effects on myocardial and cerebral protective antioxidation anti-inflammatory and anti liver fibrosis activity has been investigated. Previous studies have revealed that HSYA has anti-cancer effect in gastric adenocarcinoma[30,31]. So the four bioactive components in THSWD were determined at the mentioned optimum conditions.

CONCLUSION

This was the first report on chromatographic fingerprint analysis of THSWD and simultaneous determination of four bioactive components by UHPLC for the quality control of THSWD up to now. This method is reliable, sensitive and simple. The results demonstrate that the established method is feasible for comprehensive quality control of THSWD.

SIGNIFICANCE STATEMENTS

A simple and reliable ultra high performance liquid chromatography coupled with diode array detector method for taohongsiwu decoction was developed in this study. The results demonstrated that the established chromatographic fingerprint analysis of THSWD is doable for overall quality control of THSWD.

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