Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 705–713
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Identification and determination of the major constituents in traditional Chinese medicine Si-Wu-Tang by HPLC coupled with DAD and ESI–MS

Haijiang Zhang [a], Peng Shen [b], Yiyu Cheng [a,*]

[a] *College of Pharmaceutical Sciences, Pharmaceutical Informatics Institute, Zhejiang University, Hangzhou 310027, PR China*
[b] *The 1st Hospital Attached to College of Medicine, College of Medicine, Zhejiang University, Hangzhou 310027, PR China*

Received 20 September 2003; received in revised form 7 November 2003; accepted 14 November 2003

Abstract

An HPLC/DAD/ESI/MS method was established for the qualitative and quantitative analysis of the major constituents in Si-Wu-Tang, a traditional Chinese medicine formula. Based on the baseline chromatographic separation of most constituents in Si-Wu-Tang on hypersil C18 column with water–acetonitrile–acetic acid as mobile phase, 12 compounds including phenolic acids, phthalides and terpene glycoside were identified by online ESI–MS and the comparison with literature data and standard samples. Most of these compounds derive from *Paeonia lactiflora* and *Ligusticum chuanxiong*. Seven of them were quantitated by HPLC coupled with DAD. The validation of the method, including sensitivity, linearity, repeatability, recovery, were examined. The linear calibration curve were acquired with $R^2 > 0.99$ and LOD (S/N = 3) were between 0.75 and 5 ng. The repeatability was evaluated by intra- and inter-day assays and R.S.D. value were within ±2.38%. The recovery rates of selected compounds were in the range of 96.64–105.21% with R.S.D. less than 3.22%.

Keywords: Si-Wu-Tang; *Paeonia lactiflora*; *Ligusticum chuanxiong*; HPLC/DAD/MS; Quality control; TCM

1. Introduction

The traditional Chinese medicine (TCM), most of which are formulae, has been attracting more and more attentions for their complementary therapeutic effects to western medicines with few or no side effect [1,2]. However, although many TCM have been proven effective by modern pharmacological studies and clinical trials, their bioactive constituents and the remedial mechanism are still not well understood. So far, it is widely accepted that multiple constituents are responsible for the therapeutic effect of TCM [1]. This situation makes the quality control of TCM products very difficult.

Currently, two strategies of quality control for TCM products are mainly employed. The most widely applied strategy is to determine single or a few mark compounds, which usually are previously identified bioactive constituents, for the assessment of quality [3,4]. It gives certain quality indexes and the analysis is

* Corresponding author. Tel.: +86-571-87951138; fax: +86-571-87980668.
E-mail address: chengyy@zju.edu.cn (Y. Cheng).

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00650-2

also simplified. However, due to multiple constituents involved in the therapeutic effect, the contents of single or a few mark compounds cannot accurately reflect the quality of TCM products, let alone the batch-to-batch consistency.

Another strategy is based on the chromatographic fingerprint technology. Comparing with the previous strategy, it substitutes an aggregate HPLC peaks of the major constituents for a few mark compound as the quality indexes and was used to assess the batch-to-batch consistency of TCM products [5]. Actually, in recent years, this strategy has been gradually applied for the quality control standards of more and more TCM products in China. Nevertheless, this strategy is a "blind analysis", which lacks for the chemical information about the constituents of TCM products, so that it could not reflect the pharmaceutical activity of the TCM products. To solve this problem, the chemical studies on the major constituents of TCM products are very necessary as the complementarities.

Si-Wu-Tang, comprising four medical plants, i.e. *Paeonia lactiflora*, *Ligusticum chuanxiong*, *Angelica acutiloba* and *Rehmannia glutinosa libosch*, is one of the most widely used formulae of TCM. It has been used as the hematinic and to treat emmeniopathy for hundreds of years and widely adopted for the clinical use in China and Japan (Japanese name, Shimotsu-to). Recent studies showed that it also had antipruritic and antiflammatory activities [6,7]. According to the literatures [8–11], the main constituents in its four composition plants were demonstrated to be of several natural product groups, such as phenolic compounds, phthalides, alkaloid, terpene glycoside, iridoid glycoside, and so on. However, the special study on the profile constituents of Si-Wu-Tang formula has not been reported yet. The current quality control standard is based on the content of mark compound, i.e. paeoniflorin, an identified bioactive compounds in Si-Wu-Tang [3]. To more scientifically control the quality and the batch-to-batch consistency of Si-Wu-Tang products, the quality control standard based on the chemical identification of its major constituents and chromatographic fingerprint technology is being requested. Thereinto, the chemical identification of the major constituents is primary and indispensable. Thus, a reliable method for the qualitative and quantitative analysis of major constituents in Si-Wu-Tang is highly desirable.

HPLC and its coupled technique, especially with DAD and mass spectral methods, has been proven to be a powerful approach for the rapid identification of the constituents in botanic extracts and TCM [12–15].

In this paper, an HPLC/DAD/ESI/MS method for the qualitative and quantitative analysis of the major constituents in Si-Wu-Tang formula would be described.

2. Experiment

2.1. Instrumentation

2.1.1. HPLC–DAD analysis

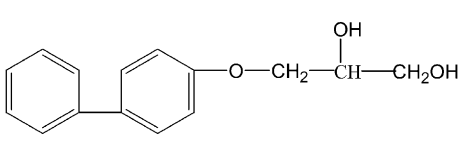
Waters-2695 Alliance HPLC instrument (Waters Corporation, Milford, MA, USA), equipped with an on-line degasser, an auto-sampler and a 2996 photodiode array detector (DAD), was used. UV detection was achieved in the scale of 210–360 nm. An Agilent Hypercil C18 column (4 mm × 250 mm, 5 μm, Serial No. US40E07157, Agilent Company, USA) was used along with Agilent C18 pre-column (4 mm × 5 mm). A linear gradient elution of A (CH_3COOH:H_2O = 0.1:100) and B (CH_3COOH:CH_3CN = 0.1:100) was used. The gradient program is presented in Table 1. The solvent flow rate was 0.8 ml/min and the column temperature was set at 30 °C.

2.1.2. HPLC–ESI–MS analysis

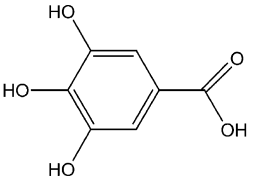
The HPLC conditions for HPLC–MS analysis were the same as those used for HPLC–DAD analysis. An Agilent 1100 series (Agilent Company, USA) LC system with DAD detection set at 230 and 280 nm was coupled to an Angilent quadrupole mass spectrometer with electrospray ionization. The ESI–MS spectra

Table 1
Solvent gradient program of HPLC analysis

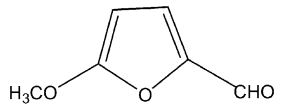
Time (min)	A (%)	B (%)
0	100	0
5	100	0
10	97.5	2.5
15	97.5	2.5
35	90	10
55	75	25
80	45	55



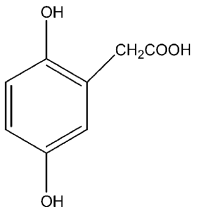
3-(4-Biphenyloxy)-1,2-propanediol (1)



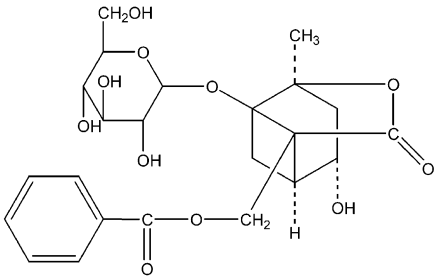
Gallic acid (2)



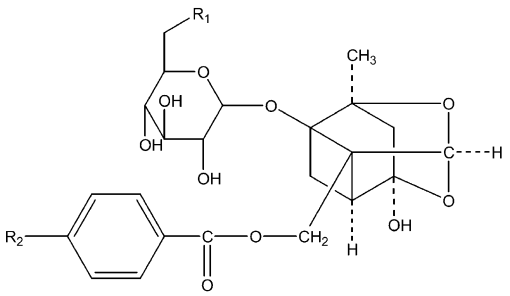
5-hydroxymethyl-2-furaldehyde (3)



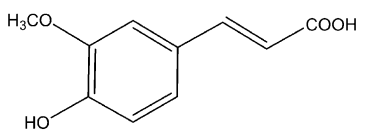
2,5-dihydroxy-phenyl acetic acid (4)



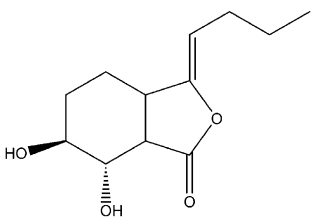
Albiflorin (5)



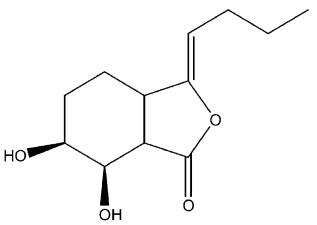
Paeoniflorin	R_1=OH	R_2=H (6)
Galloylpaeoniflorin	R_1=Galloyl	R_2=H (9)
Benzoylpaeoniflorin	R_1=$OCOC_6H_5$	R_2=H (12)



Ferulic acid (8)



Senkyunolide I (10)



Senkyunolide H (11)

Fig. 1. Structures of the constituents identified from Si-Wu-Tang.

were acquired in both the positive and negative ion mode. The conditions of HPLC–MS analysis were as follows: drying gas N_2, flow 13 l/min, gas temperature 320 °C, Quad temperature 100 °C, scan range 100–800 u, fragmentor 100, capillary voltage 3000 V.

2.1.3. Preparative HPLC

An Agilent 1100 preparative HPLC instrument (Agilent Company, USA), equipped with G-1361 dual preparative pumps and G-1315B diode array detector, was used for the preparation of standard samples.

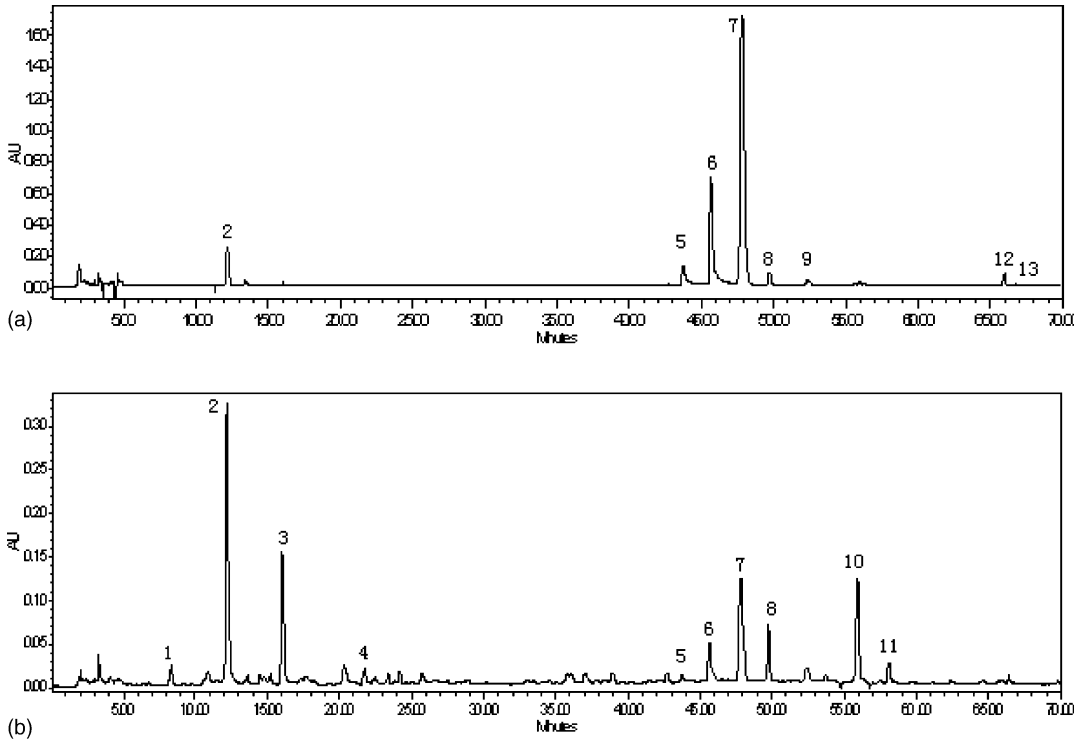


Fig. 2. HPLC chromatograms of Si-Wu-Tang at (a) 230 nm and (b) 280 nm.

The semi-preparative Agilent Zorbax SB-C18 column (9.4 mm × 250 mm, 5 μm, PN 880975202, Agilent Company, USA) was used along with Agilent C18 pre-column (10 mm). The isocratic elution methods with the mobile phase of methanol–H_2O were individually optimized before the preparation of each compound.

2.2. Regents and chemicals

Acetonitrile for HPLC analysis was of HPLC grade from Tedia (Fairfield, OH, USA); acetic acid and ethanol were of AR grade from Hangzhou Reagent Company (Hangzhou, China); water for HPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA). Twice distilled water was used for the extraction and preparation of samples.

The reference compounds paeoniflorin (6), ferulic acid (8) and ligustrazine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Gallic acid (2) was of AR grade from Shanghai Reagent Company (Shanghai, China).

2.3. Plant materials and sample preparation

Si-Wu-Tang oral solution (commercial product) were supplied from a pharmaceutical company in China. Its composition plants were purchased from Hu-qing-yu-tang pharmaceutical company (Hangzhou, China) and were identified by Professor Luan Lianjun. The voucher specimens were deposited in our laboratory.

To confirm which crude drug each ingredient in Si-Wu-Tang belongs to, the extracts of crude drugs were prepared for the HPLC analysis. The extraction process were as follows: 5 g of each plant were crushed into small pieces and were extracted with 50 ml water at 60 °C for 1 h, then followed by 50 ml 70% alcohol. The operations were repeated for three times. The total extracts were combined and the solvents were

Table 2
HPLC–DAD–ESI–MS identification[a]

Peak	Retention time (min)	Identification	$[M-H]^-$ (*m*/*z*)	$[M+Cl]^-$ (*m*/*z*)	$[M+AcO]^-$ (*m*/*z*)	$[M+M-H]^-$ (*m*/*z*)	$[M+H]^+$ (*m*/*z*)	$[M+Na]^+$ (*m*/*z*)	$[M+K]^+$ (*m*/*z*)	Crude drug	λ_{max} (nm)
1	9.03	3-(4-Biphenyloxy)-1, 2-propanediol	243	279	303	487	245	267	283	2, 3, 4	261
2	12.31	Gallic acid	169	–	–	339	171	–	–	1	214,270
3	16.5	5-Hydroxymethyl-2-furaldehyde	–	–	–	–	127	149	–	4	284
4	21.02	2,5-Dihydroxy-phenyl acetic acid	167	–	–	–	169	191	207	2	295
5	44.82	Albiflorin	479	515	539	–	481	503	519	1	231, 273
6	46.75	Paeoniflorin	479	515	539	–	481	503	519	1	232, 274
8	50.8	Ferulic acid	193	–	–	–	195	–	–	2, 3	295, 322
9	53.5	Galloylpaeoniflorin	631	667	–	–	633	655	–	1	220, 274
10	57.2	Senkyunolide I	223	259	283	–	225	247	263	2	277
11	59.5	Senkyunolide H	223	259	283	–	225	247	263	2	277
12	67.1	Benzoylpaeoniflorin	583	619	643	–	–	607	623	1	231, 274
13	67.96	Benzoylpaeoniflorin isomer	583	619	643	–	585	607	623	1	229, 274
7	49.1	Unknown compound	*m*/*z* (−): 601, 265, 121			*m*/*z* (+): 481, 365, 123				1	230, 273

[a] The number in crude drug item means which crude drug the ingredient belongs to: (1) *P. lactiflora*, (2) *L. chuanxiong*, (3) *A. acutiloba* and (4) *R. glutinosa libosch.*

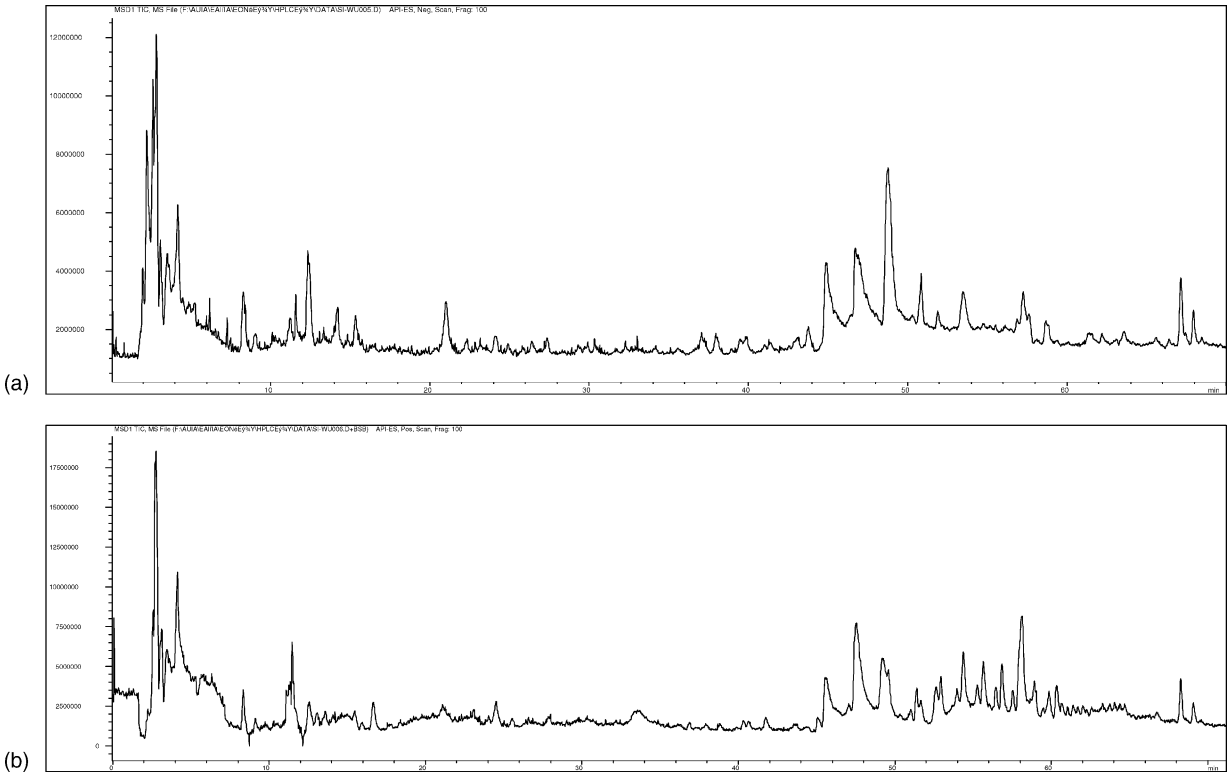


Fig. 3. MS-TIC chromatogram in (a) negative and (b) positive ion mode.

removed at 60 °C under vacuum by Buchi rotavapor B-490. The residues were then dissolved in 100 ml water to obtain the plant extracts. Si-Wu-Tang was diluted 10 times in water for quantitative analysis. All of the samples were filtered through 0.45 μm film before HPLC analysis.

Standard samples of 5-hydroxymethyl-2-furaldehyde (3), Albiflorin (5), senkyunolide I (10), as well as an unidentified compound (7), were isolated in our lab as described below.

One hundred milliliters of Si-Wu-Tang oral solution were fractionated by porous resin-101 (Tianjin Resin Company, Tianjin, China) washed with H_2O–EtOH gradient and the fractions were combined according to the HPLC analysis results. The fractions were then subjected to the semi-prepared HPLC to yield the reference samples. The identities of the isolates were characterized by ^{1}H NMR, MS spectra and the comparison with the literature data.

2.4. Validation of the method

The linearity calibration curves were made on at least eight experiments of each reference compound. The regression equation was calculated in the form of $Y = AX + B$, where Y and X were the log value of the area of peak and sample amount, respectively. The repeatability was evaluated by the intra- and inter-day ($n = 3$) assays.

Gallic acid (2), ferulic acid (8) and paeoniflorin (5) were selected as the representatives of the main types of constituent in Si-Wu-Tang, i.e. phenolic acids and terpene glycosides, and were measured the recovery. The diluted Si-Wu-Tang solution were spiked with the mixture standard samples of gallic acid (0.1017 mg/ml), paeoniflorin (0.3951 mg/ml) and ferulic acid (0.0118 mg/ml) at the ratio of 1:1, 1:2 and 1:3, respectively. Three injections of each sample were carried out for the measurement of the recovery rate.

3. Results and discussion

3.1. HPLC analysis of Si-Wu-Tang

By careful analysis of the chromatograms at different wavelengths in the scale of 210–360 nm, it was found that the chromatograms at 230 nm together with 280 nm could well represent the profile of the constituents. The representative chromatograms are shown in Fig. 1a and b. Baseline separation of the major constituents was obtained.

By comparing the chromatogram of Si-Wu-Tang with those of its composition plants' extracts, the plant derivation of each peak was confirmed in terms of the retention time and UV spectra achieved from the DAD detection. It was found that most of the peaks attributed to *P. lactiflora* and *L. chuanxiong*. Consequently, it could be assumed that these two plants are the main materials that affect the quality of Si-Wu-Tang product and should be strictly selected for the production.

3.2. HPLC–MS analysis of Si-Wu-Tang

The MS spectra were detected in both the positive and negative ion mode and their TIC chromatograms are shown in Fig. 2a and b, respectively. In MS spectra, most of constituents exhibited their quasi-molecular ions $[M - H]^-$, adducted ions $[M + Cl]^-$ and $[M + AcO]^-$ in negative ion mode, while exhibited $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ in positive ion mode. Through comparing MS spectra acquired in negative and positive ion mode, the negative ion mode was found to be more sensitive and of lower noise with the exception of peak 3 that responded only in positive ion mode. Based on the *m*/*z* value, UV spectra and the comparison with standard compounds, six peaks were unambiguously identified as gallic acid (2), 5-hydroxymethyl-2-furaldehyde (3), albiflorin (5), paeniflorin (6), ferulic acid (8) and senkyunolide I (10). Other six peaks were tentatively identified as 3-(4-biphenyloxy)-1,2-propanediol (1), 2,5-di-hydroxyphenylacetic acid (4), galloylpaeoniflorin (9), senkyunolide H (11), benzoylpaeoniflorin (12) and its isomer (13) by comparing their *m*/*z* value and UV spectra with the literature data. The results are listed in Table 2 and the structures of these compounds are shown in Fig. 3. It could be seen that the main types of the constituents in Si-Wu-Tang detected in this assay were phenolic acid, terpene glycoside, along with a few phthalides. Ligustrazine, which was reported [16] as the main active ingredients in the *L. chuanxiong*, was not detected in both *L. chuanxiong* extract and Si-Wu-Tang. It was probably lost during the course of extraction due to its easy sublimation at 22 °C. Moreover, ligustilide, an reported active compound in both *L. chuanxiong* and *A. acutiloba* [17], was detected in both crude drugs extracts while not found in Si-Wu-Tang. The peak of ligustilide in crude drugs extract was at near 80 min with HPLC program described in Table 1. It means that ligustilide is of less polarity than those in Si-Wu-Tang products. So, it is deduced it was removed in the process used in the commercial product.

For peak 7, the MS spectra in negative ion mode exhibited three peaks at *m*/*z* 601 (18), 265 (12.5) and 121 (100), respectively. By CID in directed injection mode, the fragment ions of peak *m*/*z* 601 mainly exhibited *m*/*z* 121, 341, 449, 479. In positive ion mode, the MS spectra exhibited *m*/*z* 481 instead of *m*/*z* 603. It indicated that this compound probably was

Table 3
Linearity calibration curve factors and LOD of seven constituents in Si-Wu-Tang

Peak	Compound	λ (nm)	Slope (*A*)	Intercept (*B*)	R^2	LOD (ng)
2	Gallic acid	280	1.0232	6.4845	0.9999	1.0
3	5-Hydroxylmethyl furaldehyde	280	1.0390	6.3348	0.9976	0.75
5	Albiflorin	230	1.0638	6.2386	0.9986	1.25
6	Paeoniflorin	230	1.0481	6.1238	0.9999	5.0
7	Unknown compound	230	1.0336	6.1185	0.9990	1.5
8	Ferulic acid	280	1.0175	6.6369	0.9999	1.2
10	Senkyunolide I	280	1.0473	6.4853	0.9987	1.3

Table 4
Repeatability of the method

Peak	Spiked amount (μl)	First day		Third day		Fifth day		Interdays R.S.D. (%)
		Calculated amount (μg)	R.S.D. (%)	Calculated amount (μg)	R.S.D. (%)	Calculated amount (μg)	R.S.D. (%)	
2	20	1.4764 ± 0.0083	0.57	1.4704 ± 0.0071	0.45	1.4971 ± 0.0024	0.13	0.95
3	20	0.8375 ± 0.0012	0.25	0.8474 ± 0.0042	0.46	0.8352 ± 0.0114	1.18	0.77
5	20	1.6024 ± 0.0071	0.42	1.5921 ± 0.0073	0.36	1.5812 ± 0.0115	0.77	0.69
6	20	8.4223 ± 0.0037	0.038	8.4190 ± 0.0927	1.78	8.2850 ± 0.0481	0.66	0.93
7	20	23.6480 ± 0.0919	0.34	23.3116 ± 0.1171	0.47	22.9911 ± 0.0546	0.43	1.42
8	20	0.1872 ± 0.0023	1.74	0.1963 ± 0.0026	1.18	0.1912 ± 0.0015	0.67	2.38
10	20	0.5652 ± 0.0008	0.19	0.5686 ± 0.0008	0.14	0.5693 ± 0.0018	0.42	0.39

Table 5
Recovery (%) of gallic acid, ferulic acid and paeoniflorin

Mixture ratio	Gallic acid		Ferulic acid		Paeoniflorin	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
1:1	98.87 ± 0.39	0.34	102.61 ± 0.30	0.12	105.21 ± 0.33	0.28
1:2	98.06 ± 0.32	0.32, 0.67	101.75 ± 2.03	1.74, 3.22	102.27 ± 0.40	0.36, 1.44
1:3	97.57 ± 0.23	0.64	96.64 ± 0.19	0.20	103.32 ± 0.23	0.21

of MW 602 and was derivative from paeoniflorin, which gave rise to 479 *m*/*z* as the quasi-molecular ion. However, this compound was not detected in the extract of *P. lactiflora* and also not found in the literatures and phytochemical database. Thus, it is deduced to be a new compound. The further chemical characterization of this compound is in the process and its structure would be elucidated in future report.

3.3. Validation of the method

As described in Section 2.4, the validation of the method was evaluated and the results are listed in Tables 3–5, respectively.

For all of the quantitated constituents, good linearity with $R^2 > 0.99$ were achieved. The R.S.D. ranged between 0.04 and 1.78% for intra-day assays and 0.39–2.38% for inter-day assays. The limits of detection (LOD) were between 0.75 and 5 ng. The average recovery of gallic acid, ferulic acid and paeoniflorin were 98.17% (R.S.D. 0.67%), 100.33% (R.S.D. 3.22%) and 103.60% (R.S.D. 1.44%), respectively. The similar recovery of other compounds could be expected.

4. Conclusion

This paper described a simple method for the qualification and quantitation of major constituents of Si-Wu-Tang oral solution. Twelve compounds of different types, including phenolic acid, terpene glycoside and phathalide, were identified. Another compound was deduced to be a new compound from *P. lactiflora* that has not ever been reported. Seven of these compounds were quantitated and the method presented a good sensitivity, repeatability and accuracy. *P. lactiflora* and *L. chuanxiong*, which most of the major constituents derive from, could be regarded as the main plants that determine the product quality.

This study provided the chemical support for the chromatographic fingerprint technology and facilitates to improve the quality control standard of Si-Wu-Tang oral solution.

Acknowledgements

This study was supported by TCM research plans of Zhejiang province (No. 2003C76).

References

[1] T.H. Xue, R. Roy, Science 300 (2003) 740–741.
[2] D. Normile, Science 299 (2003) 188–190.
[3] The State Pharmacopoeia Commission of PR China, Pharmacopoeia of PR China, vol. 1, Chemical Industry Press, Beijing, 2000
[4] T.R. Tsai, T.Y. Tseng, C.F. Chen, T.H. Tsai, J. Chromatogr. A 961 (2002) 83–88.
[5] Y.Y. Cheng, M.J. Chen, W.D. Tong, J. Chem. Inf. Comp. Sci. 43 (3) 1068–1076.
[6] Y. Dai, P.P.H. But, Y.P. Chan, Biol. Pharm. Bull. 25 (2002) 1175–1178.
[7] E. Tahara, T. Satoh, K. Toriizuka, J. Ethnopharmacol. 68 (1999) 219–228.
[8] X.Y. Zhang, X. Li, J. Shenyang Pharmaceut. U 19 (2002) 70–73 (in Chinese).
[9] L.Z. Lin, X.G. He, L.Z. Lian, W. King, J. Elliott, J. Chromatogr. A 810 (1998) 71–79.
[10] Y.Z. He, Z.D. Li, Foreign Med. Sci.-TCM 19 (1997) 13–17 (in Chinese).
[11] H.X. Li, M.Y. Ding, J.Y. Yu, J. Chromatogr. Sci. 40 (2002) 156–161.
[12] K. Robards, J. Chromatogr. A 1000 (2003) 657–691.
[13] X.G. He, J. Chromatogr. A 880 (2000) 203–232.
[14] Z.W. Cai, F.S.C. Lee, X.R. Wang, W.J. Yu, J. Mass Spectrom. 37 (2002) 1013–1024.
[15] H.J. Zhang, Y.J. Wu, Y.Y. Cheng, J. Pharmaceut. Biomed. 31 (2003) 175–183.
[16] M.C. Sutter, Y.X. Wang, Cardiovasc. Res. 27 (1993) 1891–1894.
[17] Q.S. Lin, Chinese Herbal Chemistry, Science Press, Beijing, 1977.