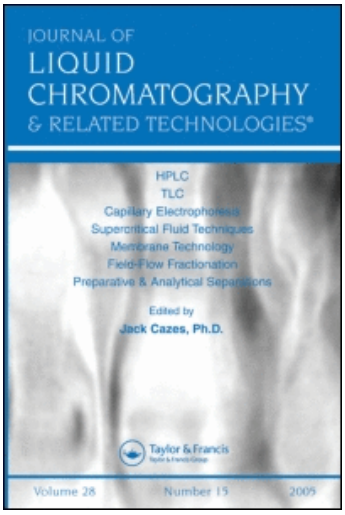

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Determination and Identification of Nine Constituents in Siho-Gyeoji-Tang by HPLC-DAD and HPLC-MS/MS

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Abstract: Simultaneous determination of four major constituents, paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin in Siho-Gyeoji-Tang (SGT) was established by an HPLC–DAD method for the quality control of traditional herbal medicinal preparation. Validation of the HPLC-DAD method showed good linearity ($r^2 > 0.997$) of the four compounds analyzed in relatively wide concentration ranges. The R.S.D. values for intra-day and inter-day precision were less than 9.0% and the limits of detection (LOD) were less than 22 ng. The mean recovery of each compound was 90.2–116.9%. In addition, analysis of ginsenosides R_{b1}, R_{g1}, saikosaponins a, b_2, and c were established by HPLC–MS in selected ion monitoring (SIM) mode, due to their low sensitivity in HPLC–DAD. These results suggest that the developed analytical method is simple and effective, and could be readily utilized as a quality control method for commercial SGT products.

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Keywords: Determination, HPLC–DAD, HPLC-MS/MS, Quality control, Siho-Gyeoji-Tang (SGT)

INTRODUCTION

Traditional herbal medicinal preparations have considerable attraction in many fields, owing to their effective therapeutic performance with minimum side effects in various diseases.[1–2] However, the quality and quantity control of their efficacy is an important issue to be solved. Traditional herbal medicinal preparations are mostly used in the combination of many herbs, which are known to be responsible for their therapeutic effects.[3] Therefore, their therapeutic effects and/or safety are affected by many factors, including cultivation environment and manufacturing process.[4–7] In addition, even though each herb has been mixed in the same ratio, different preparation procedures, such as cutting size of herbs and temperature, time, pressure used for extraction, may affect the amounts of various constituents dissolved in decoction. In other words, all these factors can affect the therapeutic effects and/or safety of traditional medicinal preparations. Therefore, the need for quality assessment of major active components in traditional herbal medicinal preparation has been increased. As such, many reports related to quality control have been carried out, mainly by the determination of major and/or active constituents.[8–11]

Siho-Gyeoji-Tang (SGT) (Chinese name; Chai-Hu-Gui-Zhi-Tang, Japanese name; Saikokeisi-to (TJ-10)) is an oral medicine and consists of 9 herb components: *Bupleurum falcatum, Pinellia ternata, Scutellaria bicalensis, Panax ginseng, Ziziphus jujube var. inermis, Zingiber officinale, Glycyrrhiza glabra, Cinnamomum cassia, Paeonia lactiflora*. It has been used in traditional medicine to relieve the symptoms of fever, pain, and other cold related disorders in Korea. SGT is a common drug to treat duodenal ulcer, pancreatitis, and chronic liver disease in Japan. Feeding a diet containing a spray dried material of SGT is known to protect against liver injuries induced by D-galactosamine-induced liver injury with reduction of an increased hepatic lipid peroxide (LPO) level in mice,[12] and gut ischemia/reperfusion-induced liver injury in rats through nitric oxide (NO) mediated inhibition of neutrophil infiltration into the liver tissue.[13] SGT has been commercially produced as granules by several medicinal manufactures. At present, the quality control of SGT is mainly conducted according to the Korea Pharmacopoeia, which quantifies only three components, paeoniflorin, baicalin, and glycyrrhizin, using each analytical method, respectively. Therefore, to ensure the efficacy and safety, a more suitable assay method for quality control has been required.

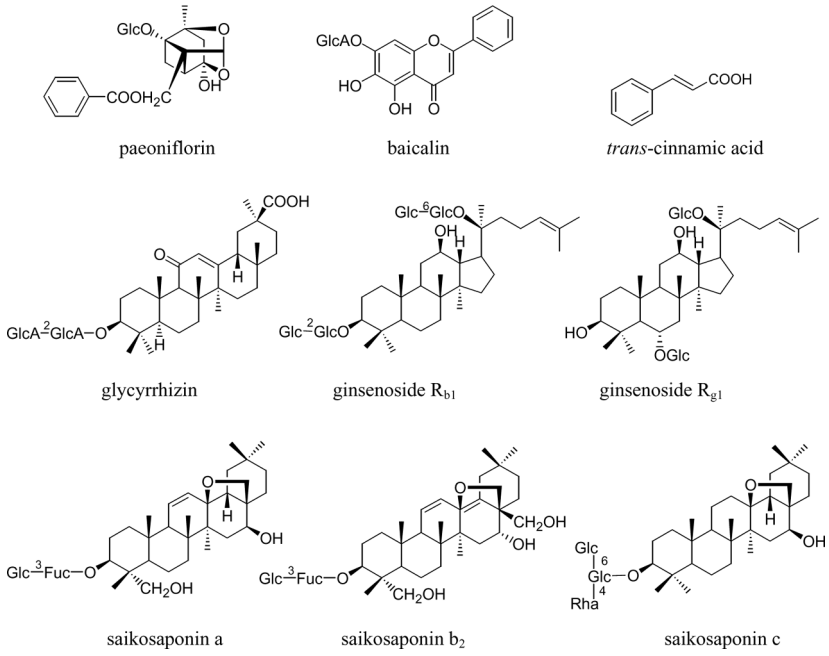

Figure 1. Structures of nine marker constituents.

In the present study, we tried to establish simultaneous determination of the major constituents of SGT, paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin (Figure 1) by HPLC–DAD. In addition, analysis of ginsenosides R_{b1}, R_{g1}, saikosaponins a, b_2, and c were established by HPLC–MS in selected ion monitoring (SIM) mode due to their low sensitivity in HPLC–DAD.

EXPERIMENTAL

Materials

All standard compounds, paeoniflorin, baicalin, *trans*-cinnamic acid, glycyrrhizin, ginsenosides R_{b1}, R_{g1}, saikosaponins a, b_2, and c were purchased from Wako (Osaka, Japan). All of the plants were purchased from Kyungdong traditional herbal market (Seoul, Korea) and were authenticated by Prof. J.H. Park in the College of Pharmacy, Pusan National University. The commercial SGT products from medicinal companies were purchased from local providers. HPLC grade solvents (acetonitrile, water, and methanol) and reagents were obtained from

BDH chemicals (Poole, UK). Phosphoric acid and acetic acid (analytical grade) were purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

Preparation of Standard Solutions

Stock standard solutions of four marker compounds were prepared in methanol at a concentration of 1 mg/mL, respectively. The appropriate amount of every standard solution was mixed and diluted with methanol.

Sample Preparation for HPLC

For the preparation of the SGT sample for the HPLC experiment, 1.67 g of *Bupleurum falcatum*, 1.33 g of *Pinellia ternata*, 0.67 g of *Scutellaria bicalensis*, 0.67 g of *Panax ginseng*, 0.67 g of *Ziziphus jujube var. inermis*, 0.33 g of *Zingiber officinale*, 0.67 g of *Glycyrrhiza glabra*, 1.00 g of *Cinnamomum cassia*, and 1.00 g of *Paeonia lactiflora*, were weighed accurately and mixed. Ten times that of water was added to mixed herbs and refluxed for 2 h at 90°C. The extract was filtered and evaporated *in vacuo*, and then suspended to 100 mL of 50% methanol. All the extract was filtered and evaporated *in vacuo*, and then suspended to 10 mL of 50% methanol. This sample solution was filtered through an 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC.

HPLC-DAD Conditions

The HPLC system consisted of a chromatographic pump (P680, Dionex, Germany) and an injector (7725i, Rheodyne, USA) equipped with Photo Diode Array (UVD 340U, Dionex, Germany). The output signal of the detector was recorded using a Dionex Chromelon™ Chromatography Data System. Chromatographic separation was achieved on a Waters XTerra™ RP18 (5 μm, 4.6 mm I.D. × 150 mm) by gradient elution of a mixture of acetonitrile and water containing 0.03% phosphoric acid (pH 2.03) at a flow rate of 1.0 mL/min. The diode-array UV/vis detector (DAD) was used for the detection and the wavelength for quantification was set at 250 nm.

HPLC-MS Conditions

A Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, and a binary pump (Hewlett-Packard, Waldbronn,

Germany) was used. Chromatographic separation was achieved on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm I.D. × 150 mm) by gradient elution of a mixture of acetonitrile and water containing 0.01% acetic acid at a flow rate of 0.8 mL/min. A post column microsplitter (Upchurch, WA, USA) was applied to restrict the flow to the mass spectrometer's source into 0.08 mL/min.

All ESI-MS and ESI-MS^n spectra were acquired using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray source and capable of analyzing ions up to m/z 2000. Mass spectrometer conditions were optimized in order to achieve maximum sensitivity. The source voltage was set to +36.5 V and the capillary temperature to 300°C. The other conditions were as follows: capillary voltage, +36.5 V; inter-octapole lens voltage, 10 V; sheath gas flow, 80 arbitrary units; auxiliary gas flow, 20 arbitrary units. Nitrogen (>99.999%) and He (>99.999%) were used as sheath and damping gas, respectively. The parent ions were isolated with an isolation width of 1 m/z units in positive mode and fragmented using collision energy of 45% for MS^2 experiments.

The mass scale was calibrated in the positive-ion mode using a solution consisting of caffeine, the tetra-peptide MRFA, and Ultramark 1621 (Sigma, St. Louis, MO, USA) solution. The Xcalibur software (Finnigan MAT) was used for the operation.

RESULTS AND DISCUSSION

HPLC-DAD Chromatographic Conditions

For the simultaneous determination of four marker components of SGT, paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin, the chromatographic condition was first investigated. Various mixtures of water and acetonitrile in combination with phosphoric acid were tested as a mobile phase. Acid is known to achieve better separation for organic compounds by depressing the tailing of the peaks.[14,15] In our chromatographic conditions, addition of phosphoric acid in water increased the resolution of the peaks. The wavelength for detection was tested at 210, 230, 250, and 280 nm by a DAD detector, and set at 250 nm where the four compounds showed the maximum absorption. The presence of paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin in this decoction was verified by comparing each retention time and UV spectrum with those of each standard compound, and spiking with authentic standards. As a result, the optimal gradient mobile phase consisting of acetonitrile-water with 0.03% phosphoric acid was subsequently employed for the analysis of SGT (Table 1), which led to good resolution and satisfactory peak shape at 250 nm (Figure 2).

Table 1. Solvent gradient conditions for HPLC–DAD

Final time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow rate (mL/min)
0	10	90	1.0
10	13	87	1.0
17	23	77	1.0
27	23	77	1.0
40	38	62	1.0
50	45	55	1.0
55	10	90	1.0
60	10	90	1.0

[a]Acetonitrile. [b]0.03% phosphoric acid (pH 2.03).

Validation of the HPLC-DAD Method

Specificity

Specificity was determined by the calculation of peak purity facilitated by the DAD detector and the corresponding computer software. The absorption spectrum of a single component varied very little at each time point in one peak, which supported specificity of each peak (Figure 2). Our results clearly showed the specificity of each peak for paeoniflorin, *trans*-cinnamic acid, schizandrin, and glycyrrhizin, respectively.

Linearity and Limits of Detection (LOD)

The linearity of paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin was calculated by seven concentrations of each compound. The regression equation and correlation coefficients (r^2) were listed in

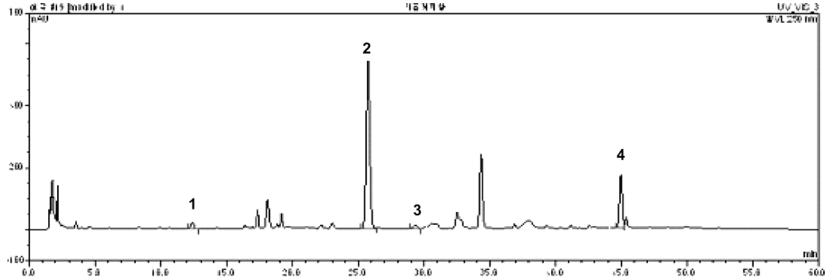

Figure 2. HPLC-UV chromatogram of SGT. 1, paeoniflorin; 2, baicalin; 3, *trans*-cinnamic acid; 4, glycyrrhizin.

Table 2. Linear ranges, limit of detection (LOD) and characteristic parameters of calibration curves for the four marker constituents by HPLC-DAD

Compounds	Linear range (μg/mL)	Linear regression equation[a] $y = ax + b$ Slope (a)	Intercept (b)	Correlation coefficient (r^2)	LOD (ng)
Paeoniflorin	0.7–325	3.9804	−0.1276	0.9968	12.0
Baicalin	1.5–500	10.036	−1.860	0.9993	22.2
trans-Cinnamic acid	0.1–125	32.794	−0.409	0.9983	2.2
Glycyrrhizin	0.6–300	12.538	−0.319	0.9997	3.1

[a] y = peak area, x = concentration (μg/ml).

Table 2, and high correlation coefficient values ($r^2 > 0.996$) showed good linearity in a relatively wide range of concentration.

The limits of detection (LOD) were measured based on the method recommended by ICH ($LOD = 3.3\,\delta/S$, δ = standard deviation of the response, S = slope of the calibration curve). LOD of paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin were 12.0, 22.2, 2.2, and 3.1 ng, respectively, which showed a high sensitivity at this chromatographic condition.

Table 3. Analytical results of intra-day and inter-day variability by HPLC-DAD

Compounds	Spiked amount (μg)	Intra-day Detected (μg)	RSD (%)	Accuracy (%)	Inter-day Detected (μg)	RSD (%)	Accuracy (%)
Paeoniflorin	3.25	3.57	3.0	90.2	3.13	6.3	103.8
	1.30	1.36	4.8	95.4	1.16	2.5	110.9
	0.65	0.70	4.3	92.3	0.60	1.7	107.7
Baicalin	15.0	15.2	0.6	98.8	14.6	1.4	102.4
	6.00	5.79	1.0	103.5	5.39	0.5	110.2
	3.00	2.72	5.2	109.3	2.49	3.0	116.9
trans-Cinnamic acid	1.25	1.29	2.5	96.9	1.20	4.0	103.4
	0.50	0.50	2.8	99.5	0.45	4.0	109.2
	0.25	0.24	9.0	106.0	0.22	0.3	113.6
Glycyrrhizin	3.00	3.05	1.6	98.2	2.98	1.7	100.8
	1.20	1.18	2.5	101.5	1.13	1.3	106.2
	0.60	0.58	4.5	103.3	0.55	1.3	108.3

Precision and Accuracy

The precision test was carried out by the intra-day and inter-day variability for these constituents. The intra-day variability was assayed at three concentrations on the same day and inter-day variability was assayed at six concentrations on three sequential days (1, 3, 5 days). As listed in Table 3, the R.S.D. (Relative standard deviation) of intra-day and inter-day variability was less than 9.0%, which demonstrated the good precision of this method. The accuracy was determined by the method of standard addition. The mean recovery of each compound was 90.2–116.9%.

HPLC-MS/MS IDENTITY CONFIRMATION

HPLC-MS in selected ion monitoring (SIM) mode was applied for the simultaneous analysis of ginsenosides R_{b1}, R_{g1}, and saikosaponins a, b_2, and c, due to their low sensitivity to UV detection and their small volume.

The sodium cationized molecule ions were shown at m/z 1131, 823, 803, 803, and 949 for ginsenosides R_{b1}, R_{g1}, saikosaponins a, b_2, and c, respectively. These parent ions were selected for identification of the five constituents because intensity of each fragment ion was very low. Under our chromatographic conditions (Table 4), all the five constituents were clearly detected in the SGT sample (Figure 3). Table 5 presents their MS and MS/MS fragment ions. Ginsenosides R_{g1} showed the $[M + Na]^+$ ions at m/z 823 in the positive ion mode. In the MS/MS spectrum, the most abundant fragment ion at m/z 643 results from loss of a H_2O and glucose

Table 4. Solvent gradient conditions for HPLC-ESI-MS

Final time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow rate (mL/min)
0	25	75	0.8
8	26	74	0.8
10	33	67	0.8
30	45	55	0.8
35	70	30	0.8
40	10	90	0.8
41	25	75	0.8
50	25	75	0.8

[a]0.01% acetic acid in acetonitrile.
[b]0.01% acetic acid in water.

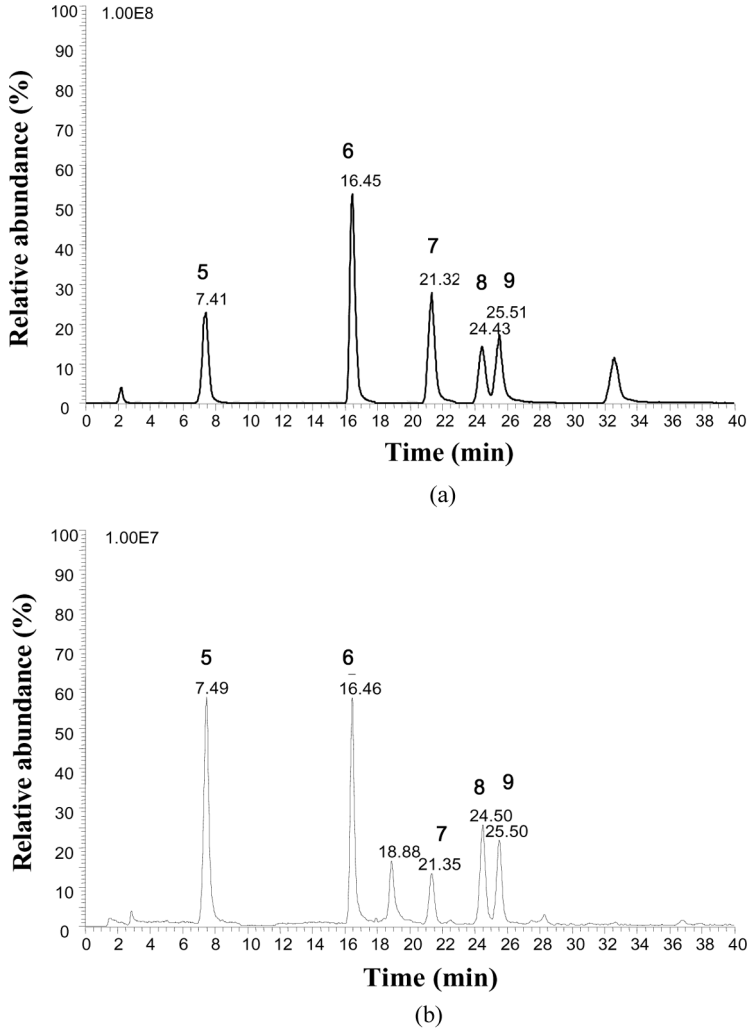

Figure 3. Total ion chromatograms of constituents of SGT in SIM mode. (a) Total ion chromatogram of the standard solution mixture (50000 ng/mL) and (b) Total ion chromatogram of SGT extract. 5, ginsenoside R_{g1}; 6, ginsenoside R_{b2}; 7, saikosaponin c; 8, saikosaponin a; 9, saikosaponin b_2. Four segments were set as follows: the ion at m/z 823 for 0–10 min, the ion at m/z 1131 for 10–18 min, the ion at m/z 949 for 18–23 min and the ion at m/z 803 for the latter period were selected for each segment.

($[M + Na–H_2O–162]^+$), which is constituent with the literature.[16] Ginsenosides R_{b1} showed the $[M + Na]^+$ ions at m/z 1131. In the MS/MS spectrum, the most abundant fragment ion at m/z 789 results

Table 5. The main fragments of constituents present in SGT determined by HPLC-ESI-MS

Compound	Precursor ion $[M+Na]^+$	MS^2 (% base peak)	Structure
5	823	683 (30), 643 (100)	ginsenoside R_{g1}
6	1131	789 (100)	ginsenoside R_{b1}
7	949	803 (100), 511 (45), 451 (20)	Saikosaponin c
8	803	331 (100)	Saikosaponin a
9	803	773 (50), 565 (20), 331 (100)	Saikosaponin b_2

from loss of a H_2O and two glucoses ($[M + Na–H_2O – 162–162]^+$), which is constituent with the literature.[16] Saikosaponin c showed the $[M + Na]^+$ ions at m/z 949 in the positive ion mode. In the MS/MS spectrum, the most abundant fragment ion at m/z 803 results from loss of a rhamnose ($[M + Na–146]^+$), and the fragment ion at m/z 511 [rhamnose + glucose + $Na]^+$ appears, which is constituent with the literature.[17] Saikosaponin a showed the $[M + Na]^+$ ions at m/z 803. In the MS/MS spectrum, the most abundant fragment ion at m/z 331 [glucose + fucose + $Na]^+$ appears, which is constituent with the literature.[15] We observed similar fragmentation patterns for the $[M + Na]^+$ (m/z 803) ion of saikosaponin b_2: product ion appeared at m/z 331 [glucose + fucose + $Na]^+$, corresponding to the mass of the sugar moiety (glucose and fucose) of saikosaponin b_2 plus a sodium ion. However, quantification was not accomplished by the flow fluctuation into MS source through the microflow splitter interface. This limit could be overcome by elimination of the flow fluctuation or changing the chromatographic conditions.

CONCLUSION

In this paper, an HPLC-DAD method for simultaneous determination of four marker constituents in SGT, paeoniflorin, baicalin, *trans*-cinnamic acid, and glycyrrhizin, has been developed and validated. The method fulfilled all the requirements to be identified as a reliable and feasible method showing good specificity, precision, linearity, and accuracy. In addition, another five bioactive components, ginsenosides R_{b1}, R_{g1}, saikosaponins a, b_2, and c were clearly identified in the SGT extract.

Therefore, this established method is useful for the quality control of SGT by simultaneous quantitative analysis of these constituents.

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