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Simultaneous Determination of Paeoniflorin, *Trans*-cinnamic Acid, Schisandrin and Glycyrrhizin in So-Cheong-Ryong-Tang by HPLC-DAD and HPLC-ESI-MS

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Abstract – High performance liquid chromatographic method with diode-array detection (HPLC-DAD) has been performed for the simultaneous determination of four marker constituents, paeoniflorin, *trans*-cinnamic acid, schisandrin and glycyrrhizin in traditional herbal medicinal preparation, So-Cheong-Ryong-Tang (SCRT). The presence of paeoniflorin, *trans*-cinnamic acid, schisandrin and glycyrrhizin in this decoction was ascertained by retention time, spiking with each authentic standard, UV spectrum and ESI mass spectrum. All four compounds showed good linearity ($r^2 > 0.998$) in a relatively wide concentration ranges. The RSD for intra-day and inter-day precision was less than 3% and the limits of detection (LOD) were less than 30 ng. The mean recovery of each compound was 94.1 – 113.0% with RSD values less than 3.0%. These results suggest that the developed HPLC method is simple, effective and could be readily utilized as a quality control method for commercial SCRT products.
Keywords – So-Cheong-Ryong-Tang (SCRT), HPLC-DAD, HPLC-ESI-MS, validation

Introduction

Traditional herbal medicinal preparations are mostly used in combination of many herbs. Multiple constituents from each herb are known to be responsible for their therapeutic effects (Xue and Roy, 2003), however, the quality of each herb has been affected by many factors such as cultivation environment and manufacturing process (Wang *et al*., 2002; Antonnen *et al*., 2006). In addition, even though each herb has been mixed in the same ratio, different preparation procedure such as cutting size of herbs, temperature, time, pressure for extraction may affect the amounts of various constituents in the decoction. In other words, all these factors can affect the therapeutic effects and/or safety of traditional medicinal preparation. Therefore, the need for quality assessment of major active components in traditional herbal medicinal preparation has been increased. As such, numerous studies related to quality control have been carried out, mainly by the determination of major and/or active constituents (Zang and Cheng, 2006; Sheng *et al*., 2005; Li *et al*., 2006).

So-Cheong-Ryong-Tang (SCRT) is combinational herbal decoction that consists of *Ephedra sinica*, *Paeonia lactiflora*, *Asarum heterotropoides* var. *mandshuricum*, *Zingiber officinale*, *Glycyrrhiza glabra*, *Cinnamomum cassia*, *Pinellia ternata* and *Schizandra chinensis*. SCRT has been used as herbal medicines for allergic diseases, such as allergic rhinitis and asthma, for hundreds of years in Asian countries (Amagaya *et al*., 2001; Kao *et al*., 2001; Ko *et al*., 2004). Untill now, several analytical methods using HPLC have been reported for each herbal component of SCRT (Jung *et al*., 1998; Okamura *et al*., 1999a; Okamura *et al*., 1999b; Wang and Yang, 2007; He *et al*., 2005; Halstead *et al*., 2007; Dong *et al*., 2007). However, there have been no reports about the simultaneous quantitative determination of their major constituents of SCRT. Thus, to ensure the efficacy and safety, a suitable assay method for quality control has been required.

In the present study, paeoniflorin (**1**), *trans*-cinnamic

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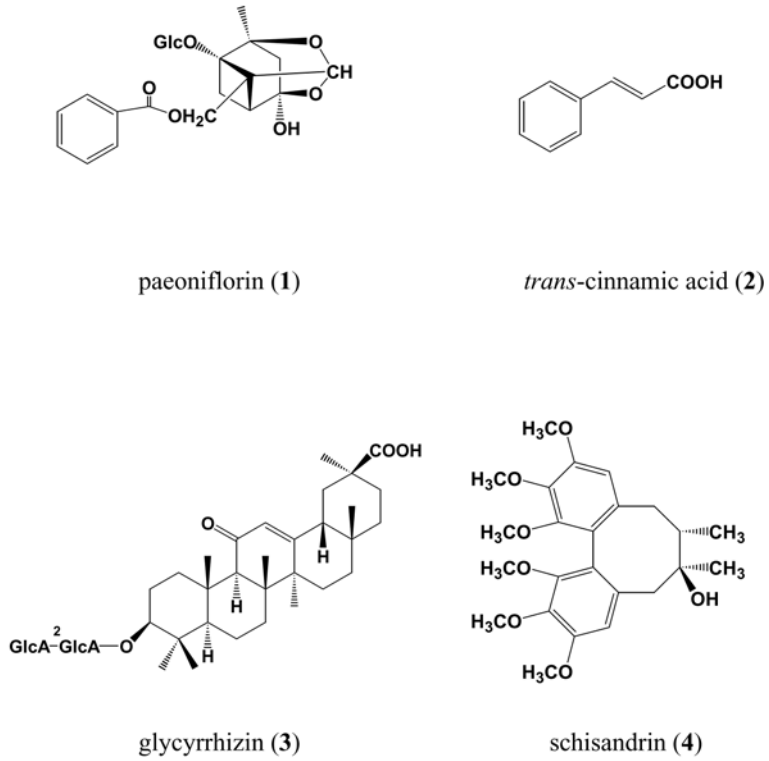

Fig. 1. Structures of four marker constituents of SCRT.

acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**), the four marker constituents of SCRT that KFDA has designated, were screened and identified by HPLC-ESI-MS technique. In addition, a HPLC/DAD method was developed and validated.

Experimental

Materials – All four compounds, paeoniflorin (**1**), *trans*-cinnamic acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**), were purchased from Wako (Osaka, Japan). All of the plants were purchased from Kyungdong traditional herbal market (Seoul, Korea) and were authenticated by Prof. Jong Hee Park in the College of Pharmacy, Pusan National University. The commercial SCRT 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 (analytical grade) was purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

Instrumentation and chromatographic conditions – The HPLC system was consisted of a chromatographic pump (P680, Dionex, Germany), 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^{TM} Chromatography Data System. Chromatographic separation was achieved on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm I.D. × 150 mm). A linear gradient elution of A (0.03% phosphoric acid) and B (100% acetonitrile) was used (0 min, 10% B; 18 min, 10% B; 40 min 30% B; 55 min, 47% B; 58 min, 10% B; 60 min, 10% B; v/v) 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-ESI-MS system consisted of Finnigan Surveyor HPLC system with a pump, an autosampler, a PDA plus detector, and Finnigan LCQ advantage MAX (ion trap mass spectrometer) with Xcalibur software. Separation was achieved on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm I.D. × 150 mm). A linear gradient elution of A (0.03% formic acid) and B (100% acetonitrile) was used (0 min, 10% B; 30 min, 40% B; 55 min 55% B; 56 min, 10% B; 60 min, 10% B; v/v) at a flow rate of 0.3 ml/min. The flow rate was 0.3 mLmin^{-1}, and the injection volume 5 μL. The conditions were as follows: capillary temperature 270 °C, i spray voltage 5 kV, sheath gas flow rate 22 arbitrary units, aux/sweep gas flow rate 0 arbitrary units.

The precursor ions were isolated with an isolation width of 2 *m/z* units and fragmented using collision energy of 40% for MS experiments.

Sample preparation for HPLC – Stock standard solution of four marker compounds was prepared in methanol at a concentration of 1 mg/ml, respectively. The appropriate amount of every standard solution was mixed and diluted as indicated.

For the preparation of SCRT sample for HPLC experiment, 1.0 g of each *E. sinica*, *P. lactiflora*, *A. heterotropoides* var. *mandshuricum*, *Z. officinale*, *G. glabra*, *C. cassia*, *S. chinensis* and 2.0 g of *P. ternata* were weighed accurately and mixed. Ten times of water (90 ml) was added to mixed herbs for SCRT 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. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 μm) and analyzed with HPLC.

Results and Discussion

For the simultaneous determination of SCRT, the chromatographic condition was first investigated. Various mixtures of water and acetonitrile in combination with phosphoric acid were tested as a mobile phase. In our chromatographic condition, 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 and set at 250 nm, where the four compounds showed the maximum absorption as measured by a DAD detector. The presence of paeoniflorin (**1**), *trans*-cinnamic acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**) 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 SCRT, which led to good resolution and satisfactory peak shape at 250 nm (Fig. 2B).

A HPLC-MS/MS experiment was also performed to confirm the identity of four marker constituents. A different solvent condition was used for this analysis due to reduce the flow rate for the MS detector. The chromatogram of the sample was comparable to that obtained for the HPLC/DAD analysis (data not shown). Table 1 presents their MS and MS/MS fragment ions. Paeoniflorin (**1**) was a major constituent of *P. lactiflora*

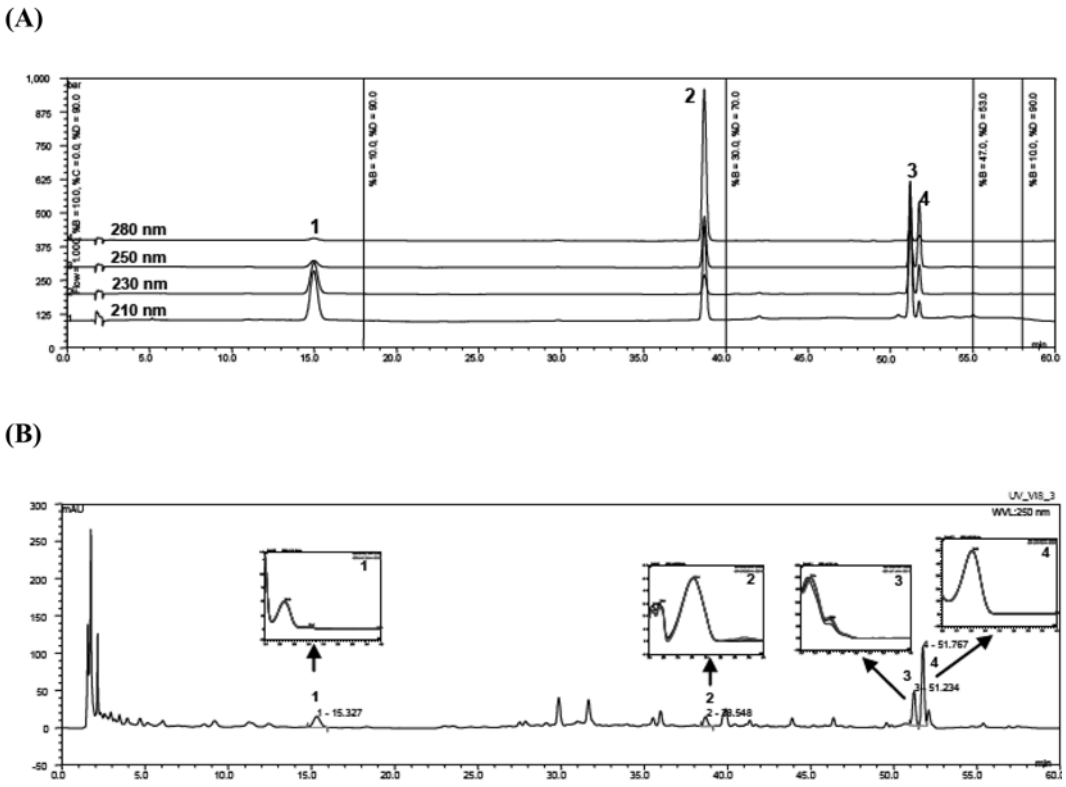

Fig 2. HPLC chromatogram of standard mixtures (A) and SCRT (B). The identification details of the peaks are shown in Table 1.

Table 1. The main fragments of four marker constituents present in So-Cheong-Ryong-Tang (SCRT) determined by HPLC-ESI-MS

Compound	ESI-MS		ESI-MS/MS
	negative mode	positive mode	
paeoniflorin (**1**)	525 $[M + CH_2O_2 - H]^{-,a}$, 479 $[M - H]^-$ (< 1% of base peak)	–	479 $[M - H]^-$ 449 $[M - H - CH_2O]^-$
trans-cinnamic acid (**2**)	147 $[M - H]^{-,a}$	–	103 $[M - H - CO_2]^-$
schisandrin (**3**)	–	433 $[M + H]^+$ (< 10% of base peak), 415 $[M - H_2O + H]^{+,a}$	384 $[M - H_2O + H - OCH_3]^+$
glycyrrhizin (**4**)	821 $[M - H]^{-,a}$	–	645 [M – H – glucuronide]$^-$

[a] precursor ion for MS/MS analysis

Table 2. Linear ranges, limit of detection (LOD) and characteristic parameters of calibration curves of four marker constituents of SCRT

Compound	Linear range (μg/ml)	Linear regression equation[a] $y = ax + b$		Correlation coefficient (r^2)	LOD (ng)
		Slope (a)	Intercept (b)		
paeoniflorin (**1**)	0.5 – 470	3.4834	0.0175	0.9985	30.0
trans-cinnamic acid (**2**)	1.2 – 120	38.1121	–0.2989	0.9994	5.2
schisandrin (**3**)	0.3 – 290	26.4394	0.0150	0.9997	5.2
glycyrrhizin (**4**)	0.1 – 120	13.2495	–0.6527	0.9981	4.1

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

and showed molecular species $[M + CH_2O_2 - H]^-$ at *m/z* 525 and the $[M - H]^-$ ion at *m/z* 479 in the negative ion mode. In MS/MS spectrum, the most abundant fragment ion at *m/z* 449 results from loss of a formic aldehyde which is constituent with the literature (Dong *et al*., 2007). *Trans*-cinnamic acid (**2**) was a major constituent of *C. cassia* and showed molecular species $[M + CH_2O_2 - H]^-$ at *m/z* 193 and the $[M - H]^-$ ion at *m/z* 147 in the negative ion mode. Schisandrin (**3**) was a major constituent of *S. chinensis* and the base peak for schisandrin (**3**) was the $[MH_2O + H]^+$ ion, where only a very small (< 10% of the base peak) $[M + H]^+$ ion was observed in positive mode (Halstead *et al*., 2007). Glycyrrhizin (**4**), which is a major triterpene saponin of *G. glabra*, displayed a molecular ion at *m/z* 821 ($[M - H]^-$) and a fragment ion at *m/z* 645 arising from the characteristic loss of a glucuronic acid group (Jong *et al*., 2006). Then using UV absorption, HPLC retention time and standard chromatogram, the structures of the four marker compounds were verified.

Specificity was determined by the calculation of peak purity facilitated by the photodiode array detector (PDA) and the corresponding computer software. The absorption spectrum of a single component remained little variable at each time point in one peak, which supported specificity of each peak (Fig. 2B). Our results clearly showed the specificity of each peak for paeoniflorin, *trans*-cinnamic acid, schisandrin and glycyrrhizin, respectively.

The linearity of paeoniflorin (**1**), *trans*-cinnamic acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**) was calculated by seven concentrations of each compound. The regression equation and correlation coefficients (r^2) were listed in Table 2 and high correlation coefficient values ($r^2 >$ 0.998) showed good linearity in relatively wide range of concentration.

Limit of detection (LOD) was measured based on the method recommended by ICH guideline Q2B (LOD = 3.3 δ/S, δ = standard deviation of the response, S = slope of the calibration curve). LOD of paeoniflorin (**1**), *trans*-cinnamic acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**) were 30.0, 5.2, 5.2 and 4.1 ng, respectively, which showed a high sensitivity at this chromatographic condition.

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 three concentrations on three sequential days (1, 3, 5 days). As listed in Table 3, the RSD of intra-day and inter-day variability was less than 3.0%, which demonstrated good precision of this method.

The accuracy of the method set up in this study was determined by the method of standard addition. The dilute sample solution (90.0 mg/ml) was spiked with the mixture standard samples of paeoniflorin (0.47 mg/ml), *trans*-

Table 3. Analytical results of inter- and intra-day precision

Compound	Amount (μg)	Intra-day		Inter-day	
		Detected (μg)	RSD (%)	Detected (μg)	RSD (%)
paeoniflorin (**1**)	9.41	9.54 ± 0.193	2.02	9.52 ± 0.141	1.48
	4.71	4.38 ± 0.027	0.62	4.30 ± 0.081	1.88
	0.94	0.99 ± 0.013	1.39	0.97 ± 0.024	2.48
trans-cinnamic acid (**2**)	2.35	2.33 ± 0.035	1.50	2.36 ± 0.043	1.82
	1.18	1.11 ± 0.027	2.41	1.10 ± 0.013	1.18
	0.24	0.24 ± 0.006	2.56	0.25 ± 0.005	2.19
schisandrin (**3**)	2.35	2.33 ± 0.032	1.37	2.37 ± 0.065	2.72
	1.18	1.13 ± 0.013	1.17	1.12 ± 0.014	1.22
	0.24	0.23 ± 0.004	1.66	0.24 ± 0.007	2.92
glycyrrhizin (**4**)	5.88	5.97 ± 0.060	1.00	5.89 ± 0.150	2.55
	2.94	2.67 ± 0.056	2.11	2.64 ± 0.041	1.57
	0.59	0.57 ± 0.014	2.55	0.59 ± 0.014	2.36

Table 4. Accuracy for the assay of four marker constituents of SCRT

Compound	Spiked amount (μg)	Measured amount (μg)	Accuracy (%)	RSD (%)
paeoniflorin (**1**)	5.45	5.90 ± 0.096	108.4	1.62
	4.75	4.48 ± 0.051	94.1	1.13
	3.75	3.83 ± 0.085	102.1	2.23
trans-cinnamic acid (**2**)	1.35	1.51 ± 0.014	111.9	0.96
	1.17	1.28 ± 0.008	109.2	0.59
	0.92	1.04 ± 0.003	113.0	0.30
schisandrin (**3**)	1.35	1.46 ± 0.035	108.2	2.38
	1.17	1.28 ± 0.008	109.3	0.59
	0.92	0.99 ± 0.019	107.5	1.95
glycyrrhizin (**4**)	3.66	4.02 ± 0.063	109.7	1.57
	3.26	3.53 ± 0.015	107.8	0.44
	2.71	2.77 ± 0.085	103.3	3.02

cinnamic acid (0.12 mg/ml), schisandrin (0.12 mg/ml) and glycyrrhiain (0.29 mg/ml) at the ratio of 10 : 10, 10 : 7 and 10:4, respectively. As listed in Table 4, the mean recovery of each compound was 94.1 - 113.0% with RSD values less than 3.0% (n = 3).

The established method has been applied to the determination of the four marker constituents in commercial SCRT granules. Three products of SCRT produced by different GMP medicinal company were used for the determination of each compound. Chromatographic separation of paeoniflorin, *trans*-cinnamic acid, schizandrin and glycyrrhizin in each product was well achieved by using the developed method (Data not shown).

In conclusion, HPLC method for simultaneous determination of four marker constituents of SCRT, paeoniflorin (**1**), *trans*-cinnamic acid (**2**), schisandrin (**3**) and glycyrrhizin (**4**), 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 data. Therefore, this established method is useful for the quality control of SCRT by simultaneous quantitative analysis of these constituents.

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