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소시호탕에서 baicalin과 glycyrrhizin 동시 분석 및 항염 활성

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Simultaneous determination of baicalin and glycyrrhizin in Sosiho-tang by HPLC and anti-inflammatory activity

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ABSTRACT

Objectives : To quantitate the main compounds and investigate the biological activity of Sosiho-tang (Xiao-Chai-Hu-Tang, SST), simultaneous determination of baicalin and glycyrrhizin, and anti-inflammatory activity were estimated.

Methods : A quantitative analysis was performed using a high performance liquid chromatography (HPLC). Reference compounds were separated on a reversed-phase column using gradient elution with water and acetonitrile each containing acetic acid at a flow rate of 1 mL/min. And the productions of nitric oxide (NO) and prostaglandin (PG)E_2 were examined by lipopolysaccharide (LPS)-treated RAW 264.7 cells in the presence of the SST. The anti-inflammatory activity of SST was investigated by carrageenin-induced paw edema in rats. The paw volume was measured at 2 and 4 hr following carrageenin-induced paw edema in rats.

Results : The correlation coefficients of the compounds showed good linearity (r^2>0.9992) over the linear range. The precisions of intra- and inter-day were less than 7.0% of relative standard deviation (RSD) values for baicalin and less than 3.5% of RDS valuse for glycyrrhizin. Recovery rates were within the range of 95.41-101.5%. The contents of baicalin and glycyrrhizin in SST were average 70.52, 6.18 mg/g, respectively. And SST exhibited inhibitory effect on NO production in LPS-treated RAW 264.7 cells but not on PGE_2 production. Oral administration of SST (1 g/kg) showed a reduction in carrageenin-induced paw edema on rats.

Conclusions : The analytical method was applied successfully to measure the contents of baicalin and glycyrrhizin in SST which exhibited anti-inflammatory activities.

Key words : Sosiho-tang (Xiao-Chai-Hu-Tang, SST), quantitative analysis, baicailn, glycyrrhizin, anti-inflammatory activity

Introduction

Traditional Korean medicines (TKMs) have treated many diseases for thousands of years in Eastern culture mostly by herbal medicines. The medicines are usually composed of several herbs containing multiple compounds. As many herbs from different location have different contents of bioactive compounds, quality control of composed herbs is important to maintain the efficacy of medicine.

Sosiho-tang (Xiao-Chai-Hu-Tang, SST) was first registered in 『Sanghan-ron (傷寒論)』 to treat alternating chills and fever, hardness and fullness in the hypochondrium, dry retching, bitter taste in the mouth.

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Recent experimental papers have demonstrated pharmacological effects of SST such as immunoregulation[1,2], anti-hepatitis[3], anti-virus[4], anti-oxidant activity[5]. It has been also clinically used to treat liver cirrhosis[6], chronic hepatitis[7], liver cancer[8], viral hepatitis[9].

Currently, many analytical methods have been available to evaluate the quality of medicinal herbs. Among them, HPLC-UV analysis was applied to qualitatively analyze three main compounds of SST such as baicalin, glycyrrhizin, saikosaponin b_2 from Scutellariae radix, Glycyrrhizae radix, Bupleurum radix, respectively[10]. And previous study[11] reported anti-inflammatory effect through *in vivo* periodontal disease model.

In the present study, we performed quantitative analysis of baicalin and glycyrrhizin in SST using high-performance liquid chromatography (HPLC) coupled with photodiode array (PDA) detection and estimated anti-inflammatory activity of SST using *in vitro* and *in vivo* model.

Materials and Methods

1. Quantitative analysis

1.1. Reagents and plant materials

HPLC-grade acetonitrile and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). Analytical reagent-grade glacial acetic acid was procured from Junsei (Tokyo, Japan). Baicalin (≥99.0%) and glycyrrhizin (≥98.0%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan, Fig. 1). The herbal materials of SST were purchased from Omniherb (Yeongcheon, Korea) and HMAX (Jecheon, Korea). It was taxonomically confirmed by Professor Je-Hyun Lee, Dongguk University, Gyeongju, Republic of Korea. Each voucher specimen (2008-KE26-1~KE26-7) was deposited at the Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine.

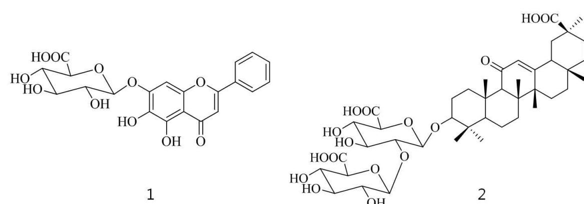

Fig. 1. Chemical structures of standard compounds. (1) baicalin; (2) glycyrrhizin

1.2. Chromatographic instrumentation and conditions

HPLC system was a Shimadzu LC-6A (Shimadzu Co., Kyoto, Japan) consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler and a PDA detector. The data acquisition was proceeded by LC Solutions (Version 1.24). The analysis was carried out on a Gemini C_{18} (250×4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA). The mobile phase was composed of solvent A (1.0% v/v aqueous acetic acid) and solvent B (acetonitrile with 1.0% v/v acetic acid). The gradient elution was applied as follows: A:B=85:15 (0 min) - A:B=60:40 (20 min) - A:B= 45:55 (40 min) - A:B=0:100 (45 min holding for 5 min). A flow rate was 1.0 mL/min with PDA detection at 254 and 280 nm. The injection volume was 10 μL.

1.3. Preparation of standard solutions

Standard compounds were dissolved in methanol at concentrations of 1 mg/mL and deposited at 4° C. A stock solutions were diluted to make a calibration curves in range of 7.81-250 μg/mL for both baicalin and glycyrrhizin.

1.4. Preparation of sample solutions

A decoction of SST was prepared with a mixture of herbal medicines according to composition in laboratory (Table 1). The herbal medicines of SST were extracted in distilled water at 100° C for 2 hr. The extract solution was evaporated to dryness and then freezed to dry powder (yield 22.9%). The accurately weighed SST extract powder (200 mg) was dissolved into a 20 mL flask and distilled water was added to the volumetric flask. Prepared sample was filtrated through a 0.2 μm syringe filter (Woongki Science, Seoul, Korea) and the filtrate was diluted to quantitate the amount of baicalin in SST prior to injection. Injection volume for HPLC analysis was 10 μL.

Table 1. The composition of herbal medicines in SST.

Herbal name	Amount(g)	Origin (Pharm)
Bupleuri Radix	11.25	Korea (Omniherb)
Scutellariae Radix	7.5	China (HMAX)
Ginseng Radix	3.75	Korea (Omniherb)
Pinelliae Tuber	3.75	Korea (Omniherb)
Glycyrrhizae Radix et Rhizoma	1.875	China (HMAX)
Zingiberis Rhizoma Crudus	3.75	Korea (Omniherb)
Zizyphi Fructus	3.75	Korea (Omniherb)
Total	36.125	

1.5. Linear regression, limit of detection (LOD) and limit of quantification (LOQ)

Linear regression analysis was performed by plotting the peak area of each standard compound on the y-axis, versus the concentration of each compound on the x-axis. The stock solutions of standard compounds were diluted to smallest concentration with methanol. The detectable smallest peaks were selected as signal, and LOD and LOQ were determined to signal-to-noise ratio of 3 and 10, respectively.

1.6. Precision and accuracy

The intra- and inter-day precisions were determined by analyzing three different concentrations of each compound (i.e., low, medium, high) spiked in sample extract three times in a day and in three successive days for inter-day. The relative standard deviation (RSD) was measured to assess precision.

The accuracy was evaluated by recovery test. The recovery test was conducted by adding three different concentrations of each known standard (i.e., low, medium, and high) to samples before extraction. Samples were extracted and analyzed using the method described above. An average recovery was calculated using the following formula: Recovery (%) = $(\text{Amount}_{\text{determined}} - \text{Amount}_{\text{original}}) / \text{Amount}_{\text{spiked}} \times 100$.

2. Anti-inflammatory activity

2.1. Cell culture

RAW 264.7 cells, murine macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand island, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco BRL) and 5.5% fetal bovine serum (FBS; Gibco BRL). Cells were incubated at 37° C, 5% CO_2 in fully humidified condition.

2.2. Cell viability

RAW 264.7 cells were plated to 96-well plates at a density of 3×10^3 cells/well, and treated with SST (2 to 500 μg/mL) for 24 hr. The cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added and incubated for 4 hr. Thereafter, absorbance was read at 450 nm on a BenchmarkPlus microplate reader (BIO-RAD, San Diego, CA, USA). Results were expressed as percent absorbance (%) within SST-treated wells compared to control wells (n=6).

2.3. Nitrite determination

The quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. RAW 264.7 cells were plated at 2.5×10^5 cells/well (48 well plate) and incubated with LPS (1 μg/mL) for 18 hr in the presence of SST (2, 10, 20, 100 and 200 μg/mL). L-N^G-monomethylalrginine (NMMA, Sigma, St. Louis, MI, USA) was used as a positive control. 50 μL of cell culture medium was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid, Promega, Madison, WI, USA). The mixture was incubated at room temperature for 30 min and the absorbance was measured at 535 nm in a microplate reader. The quantities of nitrite were determined based on sodium nitrite standard curves.

2.4. PGE_2 determination

RAW 264.7 cells were seeded at 2.5×10^5 cells/well (48 well plate) and incubated for 18 hr in the presence of LPS (1 μg/mL) and SST (2, 10, 20, 100 and 200 μg/mL), then arachidonic acid (finally 30 μM) was added. After 15 min, PGE_2 concentration in the culture medium was quantified using a competitive ELISA kit (Amersham, Little Chalfont, BU, USA) according to the manufacturer's instructions. The production of PGE_2 in cultured medium was measured relatively comparing that in control cultures.

2.5. Carrageenin-induced paw edema in rats

Four-week-old male Sprague-Dawley (SD) rats were purchased from OrientBio Inc. (Seongnam, Korea) and housed in controlled environmental conditions (22±3° C with 12/12 hrs light/dark cycle) for one week prior to the experiment. Animals were given rodent diet and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health 'Guide for the Care and Use of Laboratory Animals'.

Rats were divided into three groups (5 rats per group), control group was administrated vehicle (0.1% Tween 80) and positive control group was administrated indomethacin (10 mg/kg body weight; Sigma, Saint Louis, MI, USA) orally 3 hr prior to induced paw edema. SST group was administrated SST (1 g/kg body weight) orally for seven days prior to induction of edema. Edema was induced by subcutaneous injection of 0.1 mL of PBS containing 1% (v/v) carrageenin (Sigma, St Louis, MI, USA) into the right hind paw. Right hind paw volume was measured using a plethysmoneter (Ugo-Basile Co.,

Italy). The paw volume was measured at 2 and 4 hr following carrageenin-induced paw edema in rats.

2.6. Statistical analysis

The results were represented as means±SEM and deemed statistically significant at $p<0.05$, based on ANOVA or Bonferroni multiple comparison analysis using the Systat®program (Ver.8.0, SYSTAT Inc., Evanston, IL, USA).

Results and Discussion

1. Quantitative analysis

1.1. Optimization of chromatographic conditions

An HPLC method was successfully developed to separate the reference compounds in SST. Acetonitrile and water were selected as mobile phase and acetic acid was added to prevent the breakdown of ionic compound or tailing of compound peak. Detection was performed at maximum absorption wavelengths of each compound, 254 and 280 nm, respectively. With the PDA, UV spectra of the reference compounds in SST could be compared with those of standard compounds, therefore each reference compound was identified by comparing both the retention time and UV spectra with standard compounds. All analytes showed baseline separation and they were eluted within 40 min. Typical chromatograms of standard compounds and extract of SST are shown in Fig. 2.

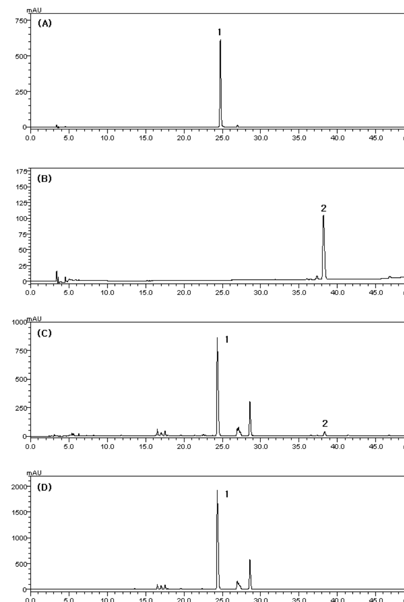

Fig. 2. HPLC chromatograms of baicalin at 280nm (A), glycyrrhizin at 254nm (B), SST extract at 254nm (C) and 280nm (D). (1) baicalin; (2) glycyrrhizin.

1.2. Linear regression, limit of detection (LOD) and limit of quantification (LOQ)

Stock solutions containing standard compounds were diluted to construct the calibration curves. At least six concentrations of solutions were analyzed with the linear range of 7.81–250 μg/mL for baicalin and glycyrrhizin. Calibration curve showed good linearity ($r^2>0.9992$). The LOD and LOQ were 0.41–1.37 μg/mL for baicalin and 0.21–0.7 μg/mL for glycyrrhizin (Table 2).

Table 2. Linear regression, correlation coefficients (r^2), LOD and LOQ of the reference compounds.

Compound	Linear equation	r^2	Linear range (μ g/mL)	LOD[a] (μ g/mL)	LOQ[b] (μ g/mL)
Baicalin	y=38806x−79724	0.9993	7.81−250	0.41	1.37
Glycyrrhizin	y=8959.4x−15143	0.9996	7.81−250	0.21	0.7

[a] LOD : Limit of Detection.
[b] LOQ : Limit of Quantification.

1.3. Precision and accuracy

The reproducibility of developed method was estimated by the precisions of samples during intra- and inter-day. The intra- and inter-day variations of baicalin were 0.37–6.02% and 0.48–6.66% of RSD values and those of glycyrrhizin were 1.86–2.5% and 1.45–4.06% of RSD values (Table 3).

Table 3. Intra- and inter-day precision of the reference compounds.

Compound	Spiked conc.[a] (μ g/mL)	Intra-day (n=3)			Inter-day (n=3)		
		Detected conc. (μ g/mL)	Precision (RSD[b], %)	Accuracy (%)	Detected conc. (μ g/mL)	Precision (RSD, %)	Accuracy (%)
Baicalin	20	20.28	6.02	101.58	20.53	6.66	103.11
	30	29.60	1.95	98.78	29.39	2.28	98.27
	45	45.14	0.37	100.36	45.17	0.48	100.58
Glycyrrhizin	12	12.39	2.52	104.87	12.38	4.06	104.30
	25	24.81	3.19	100.05	24.59	3.02	98.91
	35	35.00	1.86	100.58	35.16	1.45	100.84

[a] conc. : concentration
[b] RSD : Relative Standard Deviation (%) = (Standard Deviation / Mean) × 100.

1.4. Recovery test

To evaluate the accuracy of our method, recovery was measured by adding three different concentrations of mixed standard solutions to samples. Recovery values of the reference compounds ranged from 94.57-101.50% with a RSD less than 3.0% (Table 4).

Table 4. Recovery of the reference compounds (n=3).

Compound	Initial conc.[a] (μ g/mL)	Spiked conc. (μ g/mL)	Detected conc. (μ g/mL)	Recovery[b] (%)	RSD[c] (%)
Baicalin	70.52	20	89.95	97.13	0.85
		30	100.03	98.37	0.70
		45	115.50	99.97	0.52
Glycyrrhizin	61.75	12	73.93	101.50	1.53
		25	85.61	95.41	0.63
		35	94.85	94.57	2.40

[a] conc. : concentration
[b] Recovery (%) = (Concentration $_{detected}$ - Concentration $_{original}$) / Concentration $_{spiked}$ ×100
[c] RSD : Relative Standard Deviation (%) = (Standard Deviation / Mean) × 100.

1.5. Determination of reference compounds in SST

The developed method was successfully applied to the simultaneous determination and quantitation of the two compounds in SST. A representative chromatograms of the extracts is shown in Fig. 2 (C,D) and the contents of reference compounds are summarized in Table 5.

Table 5. The contents of the reference compounds in SST (n=3).

Compound	Contents (mg/g)	RSD[a] (%)
Baicalin	70.52±0.78	1.10
Glycyrrhizin	6.18±0.04	0.65

[a] RSD : Relative Standard Deviation (%) = (Standard Deviation / Mean) × 100.

2. Anti-inflammatory activity

2.1. Cell viability

Cell viability was not significantly altered by SST up to 200 μ g/mL (data not shown). Thus, non-toxic concentrations of SST were used for all subsequent experiments.

2.2. Inhibition of NO production but not PGE_2 in LPS-treated RAW 264.7 cells

The levels of NO increased in the culture media of LPS-treated RAW 264.7 cells but this increase was concentration-dependently inhibited by addition of SST. SST (100 and 200 μ g/mL) significantly inhibited NO production by 20.0% and 23.1% ($p<0.01$ compared to the LPS group, Fig. 3). In contrast, the levels of PGE_2 didn't show the differences between LPS-treated RAW 264.7 cell media cultured with or without SST (data not shown).

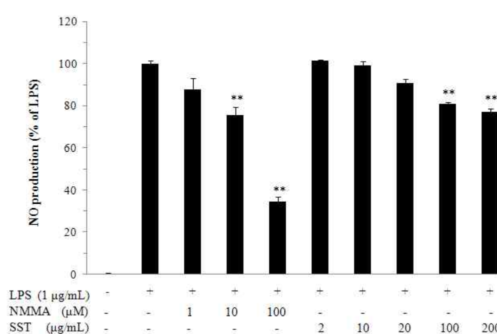

Fig. 3. Effects of SST on LPS-stimulated NO production in RAW 264.7 cells. Cells were treated with LPS (1 μ g/mL) and cultured for 18 hr with or without SST. The nitrite concentrations in cell culture supernatants were measured as described in Materials and Methods. Data are presented as mean±S.E.M. and are representative of triplicate experiments (**; $p<0.01$ compared to cells cultured with LPS).

2.3. Carrageenin-induced paw edema in rats

Pre-administration of SST (1 g/kg/day) reduced the thickness of carrageenan-induced paw edema ($p<0.01$ compared to the control group, Fig. 4).

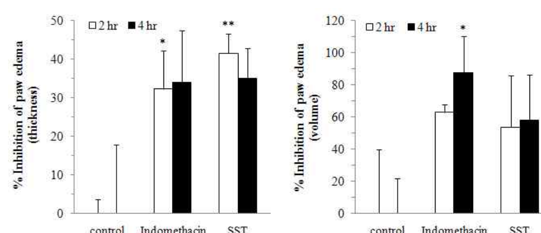

Fig. 4. Effects of SST on carrageenin-induced paw edema in rats. The control group (0.1% Tween 80) and SST group (1 g/kg) were orally administered for seven days prior to the inception of carrageenin-induced paw edema. The indomethacin group (10 mg/kg) was orally administered for 3 hr prior to the inception of carrageenin-induced paw edema. The paw volume was measured as described in Materials and Methods. Data are presented as mean±S.E.M (*; $p<0.05$ and **; $p<0.01$ compared to the control group).

Conclusion

A HPLC method for simultaneous determination of two reference compounds in SST was established. This method is simple, accurate and specific to each reference compound, and showed good linearity, precision and accuracy. Thus, the application of method was suitable and robust for the quantitative analysis of SST. And SST also could be useful for inflammatory diseases by reducing NO production and paw edema in rats.

Acknowledgments

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