Seo and Shin, Afr J Tradit Complement Altern Med., (2018) 15 (2): 88-95
https://doi.org/10.21010/ajtcam.v15i2.11

SIMULTANEOUS ANALYSIS OF THE BIOACTIVE COMPONENTS OF AN EXTRACT OF YEONGGYECHULGAM-TANG, A TRADITIONAL HERBAL PRESCRIPTION, USING HPLC–DAD

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Article History

Received: June. 29, 2017.

Revised Received: Jan. 16, 2018

Accepted: Jan. 16, 2018

Published Online: Feb. 23, 2018

Abstract

Background: Yeonggyechulgam-tang (YGCGT) is a well-known classic herbal formula and has been used clinically in Korea for the treatment of chest congestion. High-performance liquid chromatography (HPLC) analytical method coupled with diode-array detection (DAD) was performed for the simultaneous analysis of eight bioactive components, liquiritin apioside, liquiritin, coumarin, liquiritigenin, cinnamic acid, cinnamaldehyde, glycyrrhizin, and atractylenolide III in a YGCGT decoction.
Materials and Methods: For simultaneous analysis using HPLC, the eight components were separated using a Phenomenex Gemini C_{18} column (250 mm × 4.6 mm; particle size 5 μm) eluted with a gradient of 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile at 1.0 mL/min. The column temperature and injection volume were 40°C and 10 μL.
Results: Correlation coefficients of the eight compounds ranged between 0.9996 and 1.0000. The lower limits of detection and quantification of the analytes were 0.01–0.09 and 0.02–0.28 μg/mL, respectively. Recovery of the eight compounds was 97.63–102.70% and the relative standard deviation (RSD) was less than 3.00%. The RSDs of intra and interday precision were 0.06–2.07% and 0.02–1.95%, respectively. The amounts of the eight compounds in a lyophilized YGCGT were in the range 0.18 to 10.34 mg/g.
Conclusion: The optimized and validated HPLC analytical method used in the present study is expected to be useful for evaluation the quality of YGCGT decoctions or related herbal prescriptions.

Key words: Simultaneous analysis; Yeonggyechulgam-tang; HPLC–DAD; traditional herbal prescription

Introduction

Traditional herbal formulas for the prevention and treatment of varied diseases have been of increasing interest globally, particularly in East Asian countries such as Korea, China, and Japan. The formulas are composed of two or more medicinal herbs, contain many bioactive ingredients, and can be applied to a variety of diseases (Liu et al., 2008). Standardization of traditional herbal formulas is important. However, the standardization process is very difficult and requires considerable effort. Nevertheless, standardization is necessary to assure efficient quality control, coherent safety, efficacy, and stability of traditional herbal formulas (Li et al., 2008). Yeonggyechulgam-tang (YGCGT), known as Lingguizhugan-tang in Chinese and Ryokeijutsukanto in Japanese, is a well-known, classic herbal prescription consisting of four herbal medicines, including Poria Sclerotium (Polyporaceae), Cinnamomi Ramulus (Lauraceae), Atractylodis Rhizoma Alba (Compositae), and Glycyrrhizae Radix et Rhizoma (Leguminosae) in a ratio of 2:1.5:1.5:1, respectively, based on dry weight. It has been used clinically in Korea for the treatment of chest congestion (Heo, 2004). YGCGT has been reported to have a variety of biological effects and is useful for anti-inflammation (Xi et al., 2012; Wang et al., 2015), renal failure (Park et al., 2000), liver protection (Kim et al., 1999), and chronic heart failure (Fu et al., 2010; Huang et al., 2013). An analytical method to determine cinnamic acid, a marker component of Cinnamomi Ramulus in decoctions of Lingguizhugan using a reversed phase high-performance liquid chromatography (RP–HPLC) method was reported by Huang et al (2002). However, this method is not sufficient to evaluate the quality of YGCGT using HPLC, because data for the separation and simultaneous determination of its various other components are not provided. Therefore, we achieved simultaneous analysis of multiple ingredients to

assess the quality of a YGCGT decoction using a HPLC analytical method that we validated and optimized in the present study. HPLC has become one of the most widely used techniques to analyze medicinal herbs and traditional herbal prescriptions (Li et al., 2005). Generally, it has the advantage that it can be applied to the analysis of almost all components with convenience, accuracy and precision (Waksmundzka-Hajnos and Sherma, 2010; Li et al., 2005). In this study, a HPLC analytical method coupled with diode-array detection (DAD) was established for the simultaneous analysis of eight bioactive ingredients: coumarin, cinnamic acid and cinnamaldehyde (from Cinnamomi Ramulus), atractylenolide III (from Atractylodis Rhizoma Alba), and liquiritin apioside, liquiritin, liquiritigenin, and glycyrrhizin (from Glycyrrhizae Radix et Rhizoma) in a YGCGT decoction.

Materials and Methods

Plant materials

Four raw herbal medicine components of YGCGT, Poria Sclerotium, Cinnamomi Ramulus, Atractylodis Rhizoma Alba, and Glycyrrhizae Radix et Rhizoma, were purchased from an herbal medicine market, Naemome Dah (Ulsan, Korea), and identified by a pharmacognosist, Prof. Jung-Hoon Kim, School of Korean Medicine, Pusan National University (Yangsan, Korea). Sample specimens (2012–KE48-1–KE48-4) have been deposited at the K-herb Research Center, Korea Institute of Oriental Medicine.

Chemicals and reagents

The reference standards, liquiritin (99.6%), liquiritigenin (99.8%), and glycyrrhizin (99.1%) were obtained from Biopurify Phytochemicals (Chengdu, China). Cinnamic acid (99.0%) and coumarin (99.0%) were bought from Merck (Darmstadt, Germany). Cinnamaldehyde (98.0%) was purchased from Wako Chemicals (Osaka, Japan), liquiritin apioside (98.0%) from Shanghai Sunny Biotech (Shanghai, China), and atractylenolide III (99.0%) from KOC Biotec (Daejeon, Korea). The structures of these standards are shown in Figure 1. Solvents such as methanol, acetonitrile, and water for HPLC analysis were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA, analytical reagent grade) was obtained from Merck (Darmstadt, Germany).

Apparatus and conditions

HPLC system (Kyoto, Japan) consisting of two LC-20AT pumps, a DGU-20A_3 online degasser, a CTO-20A forced air circulation type column oven, SIL-20A auto sample injector, and SPD-M20A DAD was used for the simultaneous analysis of the eight components in an extract of YGCGT. The detailed HPLC conditions in this study are shown in the Table 1. The DAD was in the range of 190–800 nm and for quantitative analysis the UV absorption of each analyte was monitored at 225 nm (atractylenolide III), 254 nm (glycyrrhizin), and 275 nm (liquiritin apioside, liquiritin, coumarin, liquiritigenin, cinnamic acid, and cinnamaldehyde).

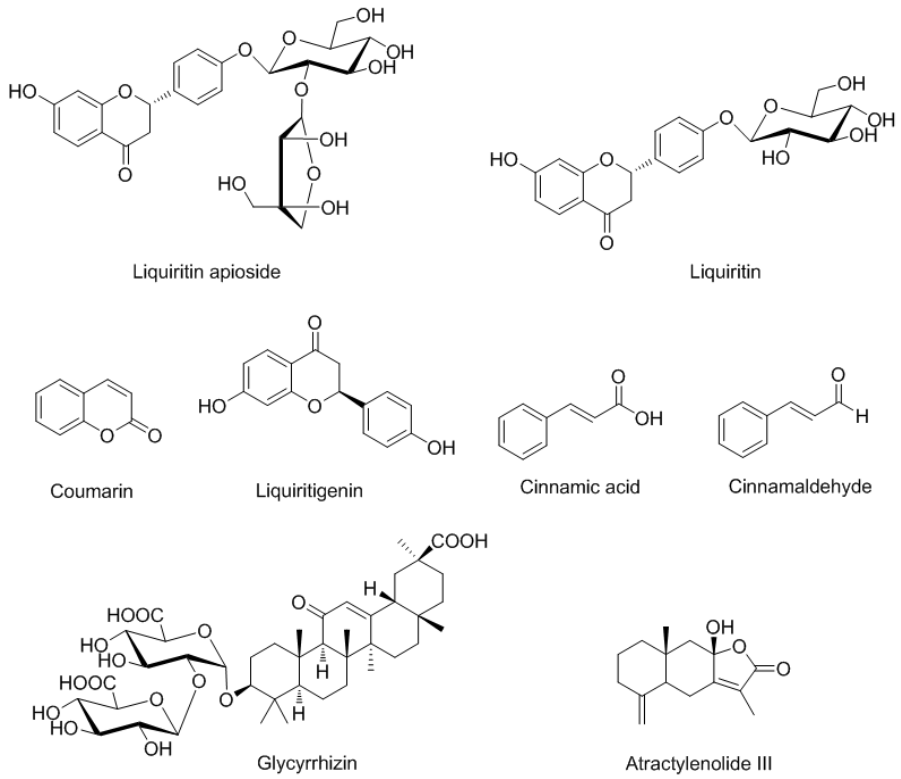

Figure 1: Chemical structures of eight bioactive compounds in Yeonggyechulgam-tang.

Preparation of reference standard solutions

Standard solutions of the eight compounds were prepared at a concentration of 1.0 mg/mL using methanol and stored at 4°C before use. Each standard solution was diluted to give a series of working standard solutions.

Preparation of YGCGT decoction and quality control sample

The YGCGT decoction consisted of four medicinal herbs as listed in Table 2 (total weight = 5.0 kg, approximately 222 times the amount of a single dose), namely the raw material from the sclerotium of *Poria cocos* (1,667 g), ramulus of *Cinnamomum cassia* (1,250 g), rhizome of *Atractylodes macrocephala* (1,250 g), and root of *Glycyrrhiza uralensis* (833 g) were mixed and extracted in 50 L of water at 100°C for 2 h. The extracted solution was lyophilized to give a powdered extract using a freeze dryer, PVT100 (IlShinBioBase, Yangju, Korea). The amount and yield of extracted YGCGT powder were 636.5 g and 12.7%, respectively. For quantitative analysis, 200 mg of freeze-dried YGCGT powder was dissolved in 20 mL of 50% methanol and then extracted using an ultrasonicator for 30 min. The extracted solution was filtered using a 0.2 μm membrane filter (PALL Life Sciences, Ann Arbor, MI, USA) before HPLC analysis.

Table 1: HPLC parameters for analysis of marker compounds in Yeonggyechulgam-tang

Operation conditions	
HPLC system	Shimadzu
Pump	LC-20AT
Detector	PDA (wavelength 225, 254, and 275 nm)
Column	Gemini C18 (Phenomenex, 250 × 4.6 mm, 5μm)
Column oven	40 ℃
Flow rate	1.0 mL/min
Auto sampler	SIL-20AC
Injection	10 μL
Mobile phase	0.1% Trifluoroacetic acid in distilled water Acetonitrile (B) 10–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min, 100–10% B for 45–50 min, and 10% B for 50–60 min.

Table 2: Composition of Yeonggyechulgam-tang.

Latin name	Scientific name	Amount (g)	Origin
Poria Sclerotium	*Poria cocos* Wolf	7.500	Pyeongchang, Korea
Cinnamomi Ramulus	*Cinnamomum cassia* Presl	5.625	Vietnam
Atractylodis Rhizoma Alba	*Atractylodes macrocephala* Koidzumi	5.625	China
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fischer	3.750	China
Total		22.500	

Calibration curves, lower limits of detection (LLOD), and of quantification (LLOQ)

A regression equation using reference standard solutions was created by plotting the peak area (y) versus the corresponding concentration (x, μg/mL) within the tested concentration ranges: liquiritin apioside and liquiritin (3.91–250.00 μg/mL), coumarin (1.56–100.00 μg/mL), liquiritigenin, cinnamic acid, and atractylenolide III (0.78–50.00 μg/mL), cinnamaldehyde (4.69–150.00 μg/mL), and glycyrrhizin (7.81–500.00 μg/mL). Samples were measured in triplicate to prepare a regression equation. The LLOD and LLOQ values were calculated by analyzing the reference standard solution using signal-to-noise (S/N) ratios of approximately 3 and 10.

Precision and recovery

To test the precision of the established HPLC–DAD method, we conducted the intra- and interday tests using a standard addition method. This method was determined by addition of three different concentrations (low, middle,

and high) of an individual reference standard to the sample. Intra- and interday precision were assessed using the relative standard deviation (RSD) as an index. Reproducibility was evaluated by measuring the quality control sample in six replicates. The RSD values of the retention times and amount of the eight compounds were used to assess the reproducibility of the established analytical method. A recovery test was performed by spiking YGCGT samples with a known concentration (low, middle, and high) of reference standard. Lyophilized YGCGT sample (200 mg) was added to a 20 mL volumetric flask and each reference standard was added at three different concentrations. HPLC-grade water was added up to the volume mark.

Results and Discussion

Optimization of chromatographic conditions

The accurate and precise HPLC method for simultaneous analysis of the eight bioactive ingredients, liquiritin apioside, liquiritin, coumarin, liquiritigenin, cinnamic acid, cinnamaldehyde, glycyrrhizin, and atractylenolide III was tested using HPLC conditions as follows: column types, including a Phenomenex Gemini C_{18} (250 mm × 4.6 mm, 5 μm), Waters SunFire C_{18} (250 mm × 4.6 mm, 5 μm), and Shiseido Capcell Pak UG120 C_{18} (250 mm × 4.6 mm, 5 μm), column temperatures (e.g., 30, 35, and 40°C), several acidic mobile phases (e.g., acetic acid, formic acid and trifluoroacetic acid), and organic solvents (e.g., methanol and acetonitrile) to optimize baseline, resolution, and peak tailing chromatographic separation. We found optimal chromatographic conditions were obtained using a Gemini C_{18} column (250 mm × 4.6 mm, 5 μm) eluted with a gradient of 0.1% (v/v) TFA in distilled water–acetonitrile at a column temperature of 40°C. To determine the eight compounds in the YGCGT decoctions quantitatively, the UV absorption wavelength was set at 225 nm for atractylenolide III, 254 nm for glycyrrhizin, and 275 nm for liquiritin apioside, liquiritin, Coumarin, liquiritigenin, cinnamic acid, and cinnamaldehyde. Figure 2 shows the typical HPLC chromatograms of reference compounds and the YGCGT sample.

System suitability

The system suitability of the established HPLC–DAD method was examined using the following parameters: capacity (k'), selectivity (α), resolution (Rs), theoretical plate number (N), and tailing factor (Tf). The findings are shown in Table 3.

Table 3: System suitability of the eight bioactive components

Compound	Capacity factor (k')	Separation factor (α)	Number of theoretical plates (N)	Resolution (Rs)	Tailing factor (Tf)
Liquiritin apioside	3.72	1.03	39811	0.97	1.06
Liquiritin	3.81	1.36	38421	0.97	1.02
Coumarin	5.18	1.11	20681	3.65	0.97
Liquiritigenin	5.77	1.06	30910	2.08	0.96
Cinnamic acid	6.10	1.10	32403	2.08	0.96
Cinnamaldehyde	6.70	1.17	25589	3.44	1.12
Glycyrrhizin	7.83	1.17	39006	6.06	1.08
Atractylenolide III	9.16	1.17	72583	8.09	0.95

Linearity, range, sensitivity, LLOD, and LLOQ

The linearity of the calibration curve was determined using correlation coefficients (r^2). The r^2 of the eight reference standards determined using this method ranged between 0.9996 and 1.0000. These findings suggest that the linearity is very good in the seven concentration ranges tested. The LLOD and LLOQ of the all analytes were 0.01–0.09 and 0.02–0.28 μg/mL, respectively, indicating good sensitivity for this analytical method. These data are summarized in Table 4.

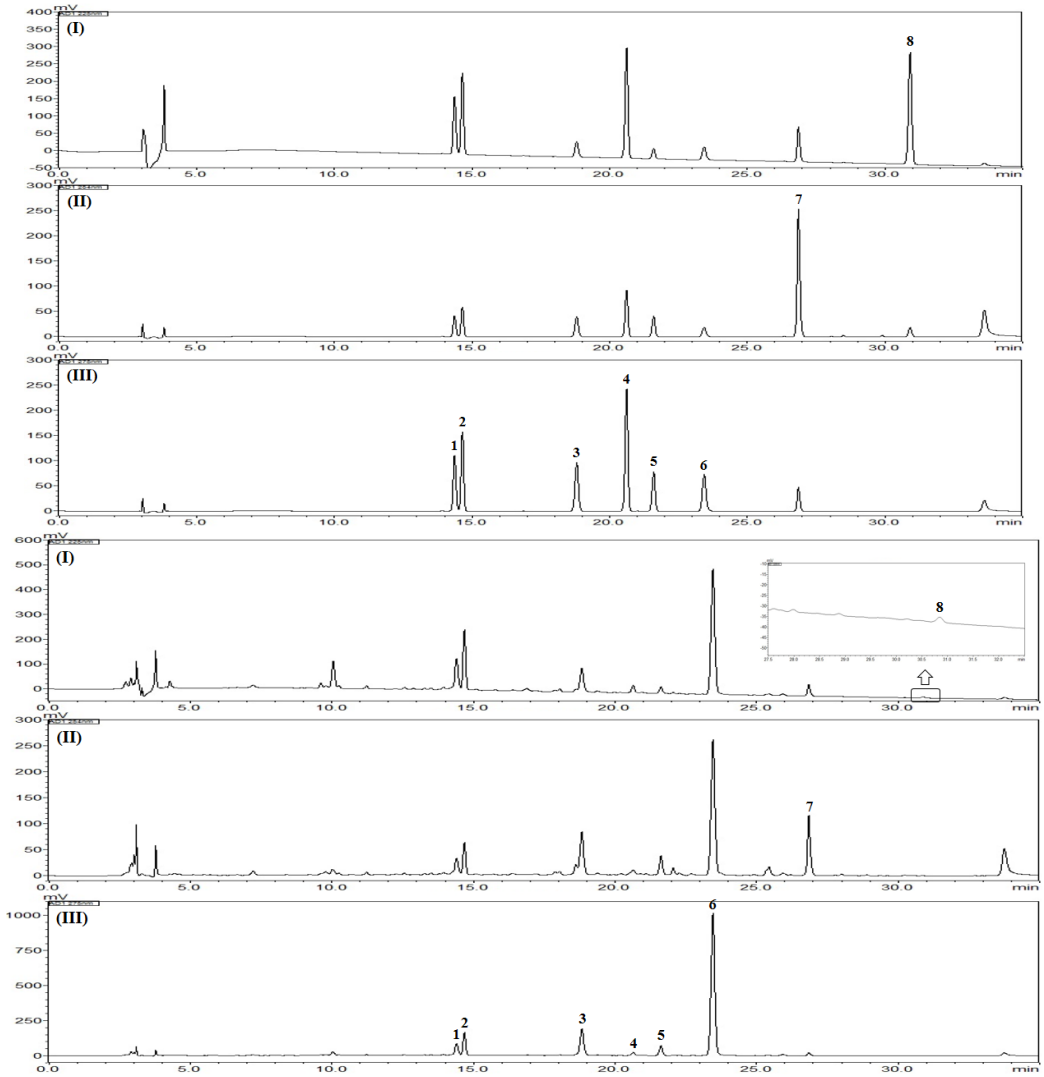

Figure 2: HPLC chromatograms of a mixture of reference standards (A) and a Yeonggyechulgam-tang sample (B) at 225 nm (I), 254 nm (II), and 275 nm (III). 1: Liquiritin apioside, 2: liquiritin, 3: coumarin, 4: liquiritigenin, 5: cinnamic acid, 6: cinnamaldehyde, 7: glycyrrhizin, 8: atractylenolide III

Table 4: Linear range, regression equations, correlation coefficients, LLODs, and LLOQs of the eight bioactive compounds

Compound	Linear range (μg/mL)	Regression equation[a]	Correlation coefficient	LLOD[b] (μg/mL)	LLOQ[c] (μg/mL)
Liquiritin apioside	3.91–250.00	$y = 15976.77x - 19465.49$	0.9999	0.05	0.15
Liquiritin	3.91–250.00	$y = 22314.06x - 3236.49$	1.0000	0.04	0.11
Coumarin	1.56–100.00	$y = 59702.66x - 29138.58$	1.0000	0.01	0.04
Liquiritigenin	0.78–50.00	$y = 34088.68x - 2776.29$	1.0000	0.02	0.07
Cinnamic acid	0.78–50.00	$y = 98964.19x - 12325.07$	1.0000	0.01	0.02
Cinnamaldehyde	4.69–150.00	$y = 138103.61x + 105800.30$	0.9996	0.01	0.02
Glycyrrhizin	7.81–500.00	$y = 8474.17x - 20042.01$	0.9999	0.09	0.28
Atractylenolide III	0.78–50.00	$y = 23958.43x - 3736.71$	1.0000	0.04	0.12

[a]y: peak area (mAU) of compounds; x: concentration (μg/mL) of compounds; [b]LLOD = 3 × signal-to-noise ratio.
[c]LLOQ = 10 × signal-to-noise ratio.

Recovery and precision

Recovery of the eight compounds from the YGCGT decoction was 97.63–102.70% and the RSD value was less than 3.00% (Table 5). Reproducibility of the optimized HPLC–DAD method was determined by the RSD as the amount of each analyte and retention time. RSD to assess reproducibility were 0.10–1.45% of the amounts of the eight compounds and 0.06–0.73% for their retention times (Table 6). The RSD of the assay for intra- and interday precision of the present method were 0.06–2.07% and 0.02–1.95%, respectively (Table 7). Therefore, the present analytical method was considered to be sufficiently accurate and precise for the simultaneous quantification of the eight bioactive compounds in YGCGT decoctions.

Table 5: Recovery test for the assay of eight compounds in Yeonggyechulgam-tang

Compound	Original conc. (μg/mL)	Spiked conc. (μg/mL)	Found conc. (μg/mL)	Recovery[a] (%)	SD	RSD (%)
Liquiritin apioside	48.97	10.00	59.24	102.62	1.23	1.20
		25.00	74.45	101.52	1.59	1.57
		50.00	99.55	101.16	0.61	0.60
Liquiritin	50.22	10.00	60.11	98.83	1.80	1.82
		25.00	75.90	102.70	2.87	2.79
		50.00	100.98	101.51	0.29	0.28
Coumarin	30.95	6.00	36.95	100.03	0.79	0.79
		15.00	46.15	101.38	0.71	0.70
		30.00	61.03	100.28	1.18	1.17
Liquiritigenin	6.22	1.00	7.23	100.77	1.08	1.08
		2.00	8.23	100.39	1.39	1.38
		5.00	11.28	101.07	0.92	0.91
Cinnamic acid	5.83	1.00	6.85	101.28	0.45	0.44
		2.00	7.86	101.41	0.43	0.43
		5.00	10.88	100.83	0.30	0.29
Cinnamaldehyde	69.63	12.00	81.50	98.84	1.15	1.16
		30.00	99.39	99.18	0.79	0.80
		60.00	129.34	99.51	0.88	0.88
Glycyrrhizin	100.88	20.00	120.64	98.82	1.49	1.51
		50.00	151.71	101.66	1.40	1.38
		100.00	201.09	100.21	0.35	0.35
Atractylenolide III	2.17	1.00	3.14	97.63	1.01	1.04
		2.00	4.15	99.21	1.56	1.57
		4.00	6.20	100.92	0.72	0.71

[a]Recovery (%) = (Found conc. – Original conc.)/Spiked conc. × 100.

Table 6: Reproducibility of retention times and amount of the eight compounds (n = 6)

Compound	Retention time (min)		Amount (μg/mL)	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Liquiritin apioside	14.26 ± 0.10	0.73	48.47 ± 0.52	1.07
Liquiritin	14.55 ± 0.09	0.60	49.71 ± 0.14	0.27
Coumarin	18.77 ± 0.01	0.07	30.72 ± 0.41	1.33
Liquiritigenin	20.58 ± 0.02	0.11	6.15 ± 0.09	1.45
Cinnamic acid	21.59 ± 0.02	0.08	5.83 ± 0.03	0.46
Cinnamaldehyde	23.42 ± 0.01	0.06	69.02 ± 0.29	0.42
Glycyrrhizin	26.84 ± 0.02	0.07	100.88 ± 0.11	0.10
Atractylenolide III	30.84 ± 0.03	0.09	2.17 ± 0.01	0.44

Table 7: Precision assay for eight compounds in Yeonggyechulgam-tang

Compound	Spiked conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)		
		Observed conc. (μg/mL)	Precision[a] (%)	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)
Liquiritin apioside	10.00	10.06	1.64	100.55	9.86	0.83	98.64
	25.00	25.10	2.07	100.41	25.15	0.95	100.60
	50.00	49.94	0.46	99.87	49.95	0.22	99.90
Liquiritin	10.00	9.76	1.71	97.62	10.10	0.59	100.99
	25.00	25.27	2.03	101.09	24.97	1.30	99.89
	50.00	49.91	0.46	99.82	49.99	0.31	99.99
Coumarin	6.00	5.95	0.50	99.23	6.06	0.61	101.05
	15.00	15.13	1.24	100.84	15.07	0.61	100.48
	30.00	29.95	0.30	99.82	29.95	0.14	99.84
Liquiritigenin	1.00	1.00	0.65	100.14	1.01	1.49	100.94
	2.00	1.99	0.93	99.54	1.97	1.43	98.30
	5.00	5.00	0.15	100.07	5.01	0.19	100.23
Cinnamic acid	1.00	1.00	0.40	100.01	0.99	1.21	99.12
	2.00	2.01	0.44	100.38	2.00	1.12	99.92
	5.00	5.00	0.06	99.94	5.00	0.14	100.05
Cinnamaldehyde	12.00	11.97	0.77	99.74	12.04	1.95	100.31
	30.00	29.95	0.74	99.82	29.64	1.18	98.81
	60.00	60.03	0.20	100.06	59.69	1.57	99.48
Glycyrrhizin	20.00	19.66	0.99	98.29	19.84	1.31	99.20
	50.00	50.59	0.82	101.19	50.54	0.27	101.09
	100.00	99.77	0.19	99.77	99.76	0.02	99.76
Atractylenolide III	1.00	0.99	0.71	98.54	0.99	0.37	99.43
	2.00	1.98	1.04	99.11	1.96	0.90	98.05
	4.00	4.01	0.23	100.31	4.02	0.20	100.52

[a]Precision is expressed as RSD (%) = (SD/mean) × 100.

Quantification of the bioactive compounds in YGCGT decoctions

The amounts of the eight compounds in the lyophilized YGCGT decoction were in the range 0.18 to 10.34 mg/g (Table 8).

Conclusion

A HPLC–DAD analytical method for the quantitative analysis of eight bioactive compound components of YGCGT decoctions was successfully established and validated, for the first time to our knowledge. We simultaneously determined eight compounds in a YGCGT decoction by using HPLC–DAD; namely, coumarin, cinnamic acid, and cinnamaldehyde (from Cinnamomi Ramulus), atractylenolide III (from Atractylodis Rhizoma Alba), and liquiritin apioside, liquiritin, liquiritigenin, and glycyrrhizin (from Glycyrrhizae Radix et Rhizoma). These eight compounds were eluted within 35 min a HPLC–DAD analytical method established and validated in the present study with a resolution of ≥0.97. The retention times of the eight analytes were approximately 14.26, 14.55, 18.77, 20.58, 21.57, 23.42, 26.84, and 30.84 min, respectively. Tests showed good linearity with an r^2 of ≥0.9996, reproducibility, recovery, and precision. Glycyrrhizin, which is a bioactive component of Glycyrrhizae Radix et Rhizoma, was determined as the most abundant bioactive compound in a YGCGT decoction. This method is expected to aid the quality control of YGCGT decoctions or related herbal prescriptions.

Table 8: The amount of eight bioactive compounds in the Yeonggyechulgam-tang (n = 3)

Compound	Batch (No.) Concentrations (mg/g) ± SD ($\times 10^{-2}$)		
	1	2	3
Liquiritin apioside	4.81 ± 0.51	4.80 ± 0.63	4.76 ± 0.31
Liquiritin	4.90 ± 0.11	4.91 ± 0.74	4.86 ± 1.22
Coumarin	3.01 ± 0.74	3.12 ± 1.32	3.04 ± 4.85
Liquiritigenin	0.54 ± 0.82	0.54 ± 0.22	0.57 ± 0.80
Cinnamic acid	0.58 ± 0.24	0.57 ± 0.04	0.57 ± 0.24
Cinnamaldehyde	6.84 ± 2.02	6.80 ± 2.07	6.79 ± 4.96
Glycyrrhizin	10.34 ± 0.91	10.30 ± 1.02	10.20 ± 2.31
Atractylenolide III	0.18 ± 0.06	0.18 ± 0.05	0.19 ± 0.06

Conflict of interest: None of the authors declare any conflict of interest related to the present study.

Acknowledgments
This study was conducted with the support of the Korea Institute of Oriental Medicine (no. K16251 and K18241).

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