Simultaneous determination of liquiritin, nodakenin and glycyrrhizinin Guibi-tang, a traditional herbal prescription by HPLC-PDA

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Abstract: A simple, convenient, rapid and accurate high-performance liquid chromatographic (HPLC) method was established for the simultaneous determination about three ingredients of a traditional herbal prescription, Guibi-tang (GBT): liquiritin (1), nodakenin (2) and glycyrrhizin (3). Chromatographic analysis on the three components was separated within 35 min on a Gemini C_{18} column and maintained at 40°C. The mobile phase consisted of water with 1.0% (v/v) acetic acid (solvent A) and acetonitrile with 1.0% (v/v) acetic acid (solvent B) in gradient mode at a flow-rate of 1.0 mL/min. Chromatograms were acquired at 254, 280 and 330 nm in a photodiode array (PDA) detector. The calibration curves showed excellent linearity (R^2=1.0000). The average recovery of three compounds was ≥93.5%, with a relative standard deviation (RSD) of ≤2.0%. The intra-day and inter-day precision (RSD) of the three components were 0.04%-3.04% and 0.11%–2.48%, respectively. The contents of compounds 1–3 in GBT were 1.09-1.10, 2.35-2.37 and 0.81-0.82 mg/g, respectively.

Keywords: Simultaneous determination; Guibi-tang; herbal prescription; HPLC–PDA; validation.

INTRODUCTION

Herbal medicines, including herbal formulas or prescriptions, have long been used in Korea, China and Japan to protect and treat varied diseases. These herbal medicines exhibit low toxicity and multiple activities (Jiang *et al.*, 2005; Liu *et al.*, 2008; Zhang *et al.*, 2004). Guibi-tang (GBT, also called Gui-pi-tang in Chinese and Kihi-to in Japanese), a traditional Korean herbal formula, is used widely for the treatment of amnesia, forgetfulness, fatigue, insomnia, anemia, palpitations and neurosis (Oh *et al.*, 2005; Tohda *et al.*, 2008). GBT is composed of 12 species of herbal medicines (table 1) and showed biological effects, for example, memory-enhancing (Oh *et al.*, 2005; Tohda *et al.*, 2008), antioxidant (Lim *et al.*, 2009), neuro protective (Lim *et al.*, 2009), protective of gastric mucosa (Kim *et al.*, 2003) and anti-stress (Eun and Song, 2007; Lee and Hwang, 1995; Lee *et al.*, 2003) effects. Recently, reports on its safety have included consideration of acute toxicity (Lee *et al.*, 2010) and reproductive and developmental toxicity (Han *et al.*, 2010). However, to data, there are no reports on the quality control of several components of GBT. For quality control, we therefore carry out the simultaneous analysis of the major components of GBT, using a high-performance liquid chromatography (HPLC) method coupled to a photodiode array detector (PDA). This analytical method is currently widely used because it is a convenient and widely applicable method for the rapid and accurately separation and analysis of multi-component in herbal medicines (Han *et al.*, 2007; Park *et al.*, 2009; Zhang *et al.*, 2004).

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In the present study, we conducted the simultaneous determination and method validation of three components of GBT, liquiritin (1), nodakenin (2) and glycyrrhizin (3) (fig. 1), using the HPLC-PDA method.

MATERIALS AND METHODS

Reagents and materials

The reference compounds1and2 were purchased from NPC Bio Technology Inc. (Daejeon, Korea). Compound 3 was obtained from Wako Chemicals (Osaka, Japan). The purity of the three standard compounds was ≥98.0% by HPLC analysis. The HPLC-grade solvents, methanol, acetonitrile and water, were obtained from J.T. Baker (Phillipsburg, NJ, USA). The glacial acetic acid (analytical reagent grade) was procured from Junsei Chemical Co., Ltd. (Tokyo, Japan).

The GBT samples used in this study consisted of 12 herbal medicines (table 1) and were purchased from Omni herb (Yeongcheon, Korea) and HMAX (Jecheon, Korea). The origins of the herbal medicines were taxonomically confirmed by Prof. Je-Hyun Lee at Dongguk University, Gyeongju, Republic of Korea, Prof. Young-Bae Seo at Daejeon University, Daejeon, Republic of Korea. A voucher specimen (2008-KE22-1~KE22-12) has been deposited at the Basic Herbal Medicine Research Group, Korea Institute of Oriental Medicine.

Preparation of reference standard solutions

Stock solution of containing 1 mg/mL of the three compounds was made in methanol and stored at 4°C.The standard solutions were serially diluted with methanol to obtain working standard solutions.

Table 1: Composition of Guibi-tang

Scientific name	Herbal medicine	Amount (g)	Supplier	Source
Angelica gigas	Angelicae Gigantis Radix	3.75	Omniherb	Yeongcheon, Korea
Dimocarpus longan	Longan Arillus	3.75	HMAX	Vietnam
Zizyphus jujuba	Zizyphi Semen	3.75	HMAX	China
Polygala tenuifolia	Polygalae Radix	3.75	HMAX	China
Panax ginseng	Ginseng Radix Alba	3.75	Omniherb	Geumsan, Korea
Astragalus membranaceus	Astragali Radix	3.75	Omniherb	Jeongseon, Korea
Atractylodes japonica	Atractylodis Rhizoma Alba	3.75	Omniherb	China
Poria cocos	Hoelen Cum Radix	3.75	HMAX	China
Aucklandia lappa	Aucklandiae Radix	1.875	HMAX	China
Glycyrrhiza uralensis	Glycyrrhizae Radix	1.125	HMAX	China
Zingiber officinale	Zingiberis Rhizoma Crudus	6.25	Omniherb	Yeongcheon, Korea
Zizyphus jujuba	Zizyphi Fructus	3.75	Omniherb	Yeongcheon, Korea
Total amount (g)		43.00		

Preparation of GBT extracts and sample solutions

The mixture of 12 crude herbs (table 1, total weight 10.0 kg) was extracted in 100 L of distilled water at 100°C for 2 h in an herb extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). After filtration using a standard sieve (No. 270, 53 μm), the solution was evaporated under reduced pressure and freeze-dried to about 2.43 kg of extract powder. The yield of the GBT extract was 24.3%. For HPLC simultaneous determination, lyophilized GBT powder (400 mg) was dissolved in distilled water (20 mL). The solution was filtered through a 0.2 μm membrane filter (Woongki Science, Seoul, Korea) before HPLC analysis.

Table 2: Composition of mobile phase for chromatographic separation

Time (min)	Solvent A (%)[a]	Solvent B (%)[b]
0	90	10
30	30	70
35	0	100
40	0	100
45	90	10
60	90	10

[a]1.0% (v/v) acetic acid in water.
[b]1.0% (v/v) acetic acid in acetonitrile.

HPLC analysis

The HPLC system consisted of aLC-20AD pump, DGU-20A3 online degasser, CTO-20A column oven, SIL-20ACautosample injector with cooler and SPD-M20APDA detector (Shimadzu Corp., Kyoto, Japan). The quantitation was based on peak area integrated by LC solution software package v.1.24 (Shimadzu Co., Kyoto, Japan). The three components in GBT were separated by using a Gemini C_{18} analytical column (250 × 4.6mm, 5 μm; Phenomenex, Torrance, CA, USA). The column oven temperature was achieved at 40°C and monitored at 254 nm for compound 3, 280 nm for compound 1 and 330 nm for compound 2. The mobile phases consisted of water with 1.0% (v/v) acetic acid (solvent A) and acetonitrile with 1.0% (v/v) acetic acid (solvent B) at a flow rate of 1.0 mL/min. The gradient conditions changed according to the table 2. The injection volume used was 10 μL.

Linearity and limits of detection and quantification

Calibration curves of compounds 1-3 were calculated from the peak areas of standard solutions in the following concentration range: compounds 1 and 3, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00 μg/mL and compound 2, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 μg/mL. Limit of detection (LOD) and limit of quantification (LOQ) for compounds 1-3 were estimated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.

Precision and accuracy

Reproducibility was determined by analyzing six independently prepared standard solutions. The relative standard deviation (RSD) of the analyte peak areas and peak retention times for each standard were calculated. The precision (Intra-day and inter-day) values were established using a standard addition method to prepare spiked samples, employing both standards and controls. The accuracy of the HPLC method was estimated from the recovery tests. The recovery tests were performed adding known concentrations (low, medium and high) of the reference standards to the GBT samples. The average recovery value was calculated using the following formula:

$$\text{Recovery}\,(\%) = \frac{(\text{Amount}_{\text{determined}} - \text{Amount}_{\text{original}})}{\text{Amount}_{\text{spiked}}} \times 100$$

Table 3: Linearity, correlation coefficient, LOD, and LOQ data for analysis of the marker compounds (n = 4)

Compound	Linear range (μg/mL)	Regression equation[a]	Correlation coefficient (R^2)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
1	0.78-100.00	Y=15,897.83x+5,924.89	1.0000	0.04	0.15
2	0.39-50.00	Y=18,956.72x+11,765.16	1.0000	0.16	0.53
3	3.13-400.00	Y=12,654.62x+3,088.20	1.0000	0.21	0.71

[a]Y = peak area (mAU) of the components, x =concentration (μg/mL) of the components.
[b]LOD = 3×signal-to-noise (S/N) ratio.
[c]LOQ = 10×signal-to-noise (S/N) ratio.

Table 4: Reproducibility of the three compounds on retention time and peak area

No. (#)	Retention time (min)			Peak area (mAU)		
	1	2	3	1	2	3
1	14.28	15.21	30.70	1,562,072	1,835,807	2,512,611
2	14.27	15.21	30.70	1,586,077	1,843,522	2,548,846
3	14.28	15.21	30.71	1,563,010	1,833,710	2,514,557
4	14.27	15.20	30.71	1,561,392	1,809,122	2,511,490
5	14.27	15.20	30.72	1,566,558	1,819,381	2,517,466
6	14.27	15.20	30.71	1,562,638	1,832,644	2,512,348
Mean	14.27	15.20	30.71	1,566,957.83	1,829,031.00	2,519,553.00
SD	0.003	0.004	0.006	9,537.281	12,492.725	14,508.693
RSD (%)	0.021	0.026	0.020	0.609	0.683	0.576

Table 5: Precision of the three marker compounds in Guibi-tang

Compound	Spiked conc. (μg/mL)	Intra-day (n=3)				Inter-day (n=3)			
		Observed conc. (μg/mL)	SD	Precision (RSD %)	Accuracy (%)	Observed conc. (μg/mL)	SD	Precision (RSD %)	Accuracy (%)
1	8.00	8.44	0.06	0.76	105.45	8.34	0.06	0.68	104.26
	25.00	24.97	0.13	0.54	99.86	25.11	0.12	0.46	100.46
	50.00	49.95	0.07	0.15	99.89	49.89	0.05	0.11	99.78
2	8.00	7.56	0.23	3.04	94.55	7.85	0.19	2.48	98.18
	50.00	53.00	0.89	1.68	106.00	51.51	0.68	1.32	103.01
	100.00	98.53	0.43	0.44	98.53	99.26	0.32	0.33	99.26
3	8.00	8.10	0.04	0.53	101.20	8.04	0.09	1.14	100.48
	25.00	25.38	0.04	0.15	101.52	25.44	0.11	0.44	101.74
	50.00	49.80	0.02	0.04	99.59	49.72	0.08	0.15	99.44

RESULTS

Optimization of HPLC separation

We obtained satisfactory results using mobile phases consisting of 1.0% (v/v) acetic acid (solvent A) and acetonitrile with 1.0% (v/v) acetic acid (solvent B). Quantitation was achieved using PDA detection in the region 190-400 nm, based on retention times and UV spectra compared with those of the standards. The UV wavelength was recorded at 254 nm for compound 3, 280 nm for compound 1 and 330 nm for compound 2. Using these optimized chromatography conditions, the three components were eluted within a period of 35 min. The retention times of compounds 1-3 were 14.27, 15.20 and 30.71 min, respectively. Fig. 2 showed the typical chromatograms of standard solutions and the extract solutions.

Linearity, LOD and LOQ

The linearity was established by linear regression analysis. Calibration curves of compounds 1-3 were obtained using standard solutions and calculated by

Table 6: Recovery levels of the three compounds in Guibi-tang

Compound	Original conc. (μg/mL)	Spiked conc. (μg/mL)	Detected conc. (μg/mL)	Recovery (%)	SD	RSD (%)
1	21.08	8.00	29.17	101.10	0.78	0.77
		25.00	44.79	94.84	0.51	0.54
		50.00	67.87	93.57	0.76	0.82
2	47.38	8.00	55.59	102.56	1.23	1.19
		50.00	97.85	100.94	1.03	1.02
		100.00	144.10	96.72	0.80	0.82
3	16.41	8.00	24.53	101.56	1.77	1.75
		25.00	41.55	100.58	0.65	0.64
		50.00	65.31	97.81	0.48	0.49

Table 7: Analytical results (mg/g) of the three compounds in Guibi-tang

Batch (#)	Content (mg/g)								
	Liquiritin (1)			Nodakenin (2)			Glycyrrhizin (3)		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
1	1.09	0.016	1.428	2.35	0.044	1.852	0.81	0.005	0.640
2	1.09	0.010	0.906	2.35	0.035	1.484	0.81	0.003	0.311
3	1.10	0.009	0.781	2.37	0.027	1.161	0.82	0.003	0.361

regression analysis of the peak area (Y) against the concentration (×, μg/mL). All the correlation coefficients (R^2) of determination of the calibration curves for the three constituents were 1.0000. These results show that there is an outstanding correlation between peak area and concentration for the three compounds. The linear equations and coefficients (R^2) of determination of the calibration curves are summarized in table 3.The LOD and LOQ values were in the range 0.04-0.21 μg/mL and 0.15-0.71 μg/mL, respectively (table 3).

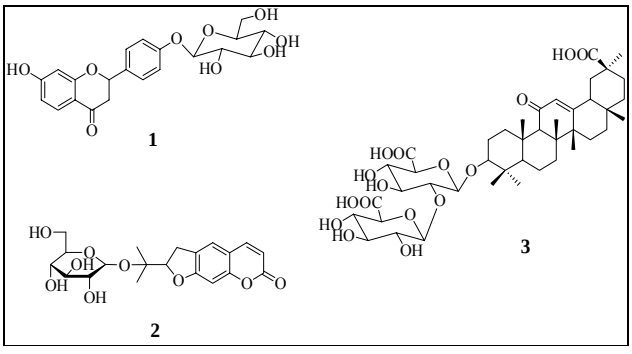

Fig. 1: Chemical structures of three constituents in Guibi-tang.

Precision and accuracy

The reproducibility on retention times and peak areas was evaluated by repeatedly measuring the standard solution. The RSD values of reproducibility for three compounds were <1.0% for peak areas and <0.03% for the retention times (table 4), indicating that this method is considerably stable. The RSDs of the intra-day and inter-day precisions about the three major constituents, liquiritin, nodakenin and glycyrrhizin, were in the range 0.04%-3.04% and 0.11%-2.48%, respectively (table 5). The intra-day accuracy was found to be 94.55%-106.00%. The inter-day accuracy was found to be 98.18%-103.01%.

The average recovery of the assay for the compounds1-3 was >93.5% and the RSD was <2.0% (table 6). These data were suggested that the newly developed analytical method was considerably precise and accurate.

Quantitativeanalysis

The newly developed HPLC-PDA method can be used to the simultaneous analysis of the three components of GBT: liquiritin, nodakenin and glycyrrhizin. Fig. 2 shows typical HPLC chromatograms of the standard solution and a GBT extract, with detection of eluents at 254, 280 and 330 nm. The contents of each compound in GBT were 1.09-1.10, 2.35-2.37 and 0.81-0.82 mg/g, respectively (table 7).

DISCUSSION

Traditional herbal medicines typically contain multiple herbs, which each contain several ingredients, and have been widely used to prevent and treat a variety of diseases. Generally, HPLC has widely been for quality control of herbal prescriptions for a long time. In previous studies, analytical methods have been reported for simultaneous determination of GBT (Liang *et al.*, 2011; Liang *et al.*, 2012). These studies focus on angiotensin-

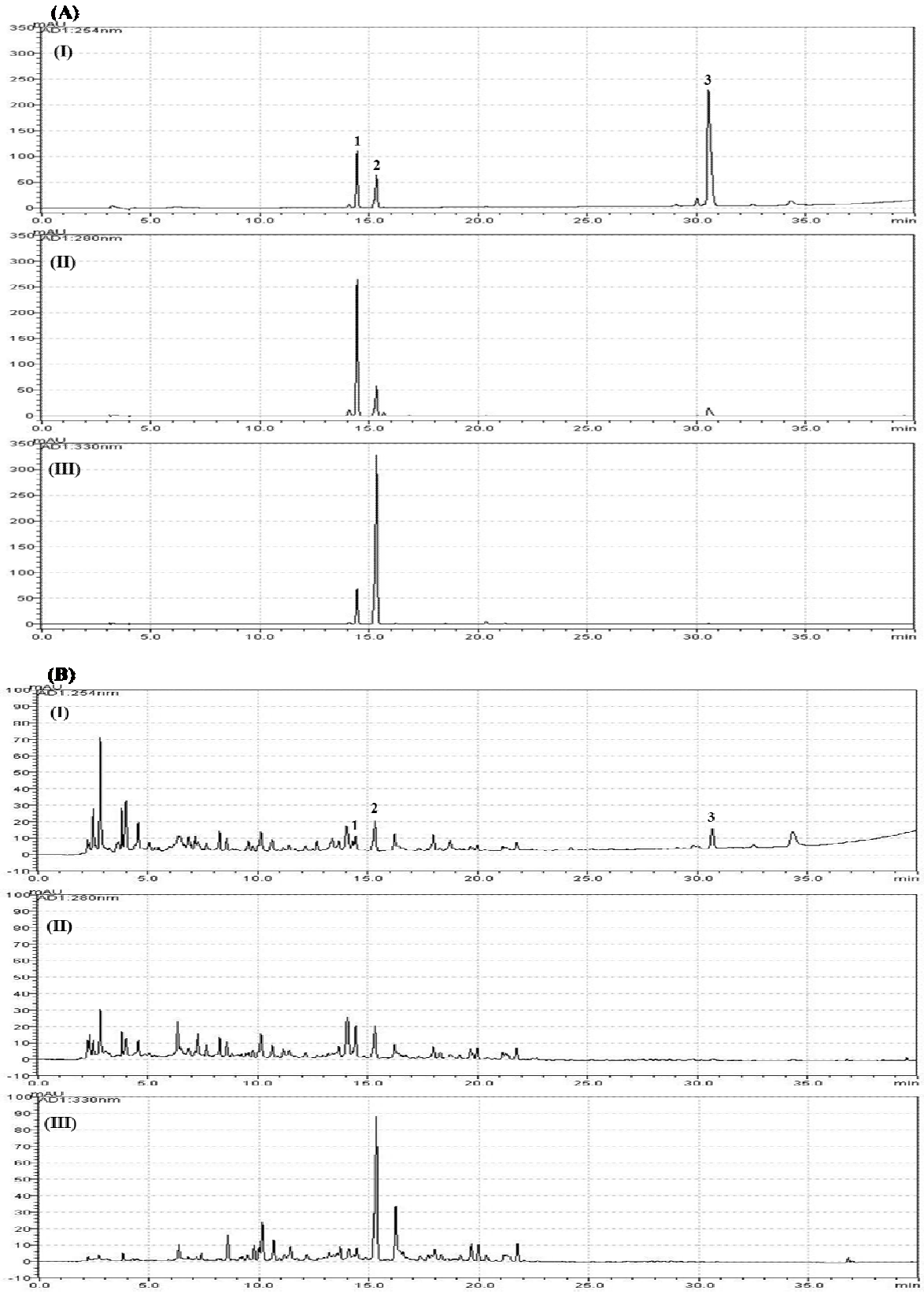

Fig. 2: HPLC chromatograms of a standard mixture (A) and Guibi-tang extract (B). 254 nm (I), 280 nm (II) and 330 nm (III). Liquiritin (1), nodakenin (2) and glycyrrhizin (3).

converting enzyme inhibitory activity and contents of fermented GBT without method validations such as linearity, precision and accuracy. However, the current method was performed method validations for verification of analysis method in simultaneous analysis. The developed method has been successfully applied to simultaneous analysis of marker constituents in GBT. Among these components, nodakenin, which is marker components of Radix Angelicae Gigantis, was found to be 2.35-2.37 mg/g and was the most abundant compounds compared with the others in the GBT extract.

CONCLUSIONS

A simple, convenient and rapid HPLC assay developed to simultaneous identification of three components of GBT and evaluated their quality control in GBT. For verifying the simultaneous determination of liquiritin, nodakenin and glycyrrhizin in GBT, we were conducted validation such as linearity, precision and accuracy. Our assay would be very useful for improving the quality control and analysis of GBT samples.

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