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Short communication

Quality control of a herbal medicinal preparation using high-performance liquid chromatographic and capillary electrophoretic methods

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ABSTRACT

Two methods based on high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were developed for the quality control of "samgiumgagambang" (SGMX), a new herbal medicinal preparation containing 14 herbs. Nine components from SGMX were selected as markers: 5-hydroxymethylfuraldehyde, geniposidic acid, chlorogenic acid, paeoniflorin, 20-hydroxyecdysone, coptisine, berberine, luteolin, and glycyrrhizic acid. The markers were identified and analyzed using HPLC coupled with a UV-diode-array detector and monitored at 250 nm with a gradient elution of acetonitrile and water containing formic acid on a C_{18} analytical column or using CE with a 70 mM borate buffer (pH 9.5) containing 10% methanol on a 60-cm fused silica capillary monitored at 230 nm. The marker components in SGMX were well separated using both methods and were readily determined within 60 min using HPLC or 13 min using CE with good precision and accuracy.

1. Introduction

Quality control of herbal medicine is an essential prerequisite for efficacy and toxicity studies [1]. Traditional herbal formulae are significantly different compared with conventional pharmacological drugs because they are mixtures of medicinal plants, which vary depending on harvesting, storing, processing, and formulating methods, and this variation affects the quality and consistency of the final product [2,3]. Thus, quality control of these multi-herbal drugs is difficult or even unfeasible. Development of quality control methods for herbal drugs typically requires that several characteristic components are identified and quantified, which involves labor-intensive analytical techniques and protocols, including chromatographic methods, such as high-performance liquid chromatography (HPLC), high-performance thin layer chromatography (HPTLC), gas chromatography (GC), and capillary electrophoresis (CE) [4,5]. Among these, HPLC is the most popular method, and liquid chromatography coupled with a diode-array detector (DAD) and mass spectrometer (MS) is a powerful analytical tool for analyses of known and unknown compounds in a complex mixture. Thus, this technique is ideally suited for herbal drug analyses [6]. In recent years, CE has been recognized as an important alternative or complementary tool in the field of herbal drug analysis [7,8]. CE is an economical technique that has many advantages, such as small injection sample volume, high efficiency, and short analysis time, which can be useful in the rapid and efficient determination of active components in complex systems [9].

A new herbal medicinal preparation, "samgiumgagambang" (SGMX) was developed by altering the herbal composition of herbs in samgium and has been used at the Daejeon University Oriental Hospital since 2001 for treating cerebral vascular damage, hypertension, and hyperlipidemia [10,11]. The efficacy of SGMX has been clinically and experimentally evaluated [12,13]. Many recent reports have described new biological activities of certain SGMX constituents, based on modern monitoring methods [14–17]. In this study, nine bioactive SGMX components, 5-hydroxymethylfuraldehyde (1), geniposidic acid (2), chlorogenic acid (3), paeoniflorin (4), 20-hydroxyecdysone (5), coptisine (6), berberine (7), luteolin (8), and glycyrrhizic acid (9), were selected for analysis. These components included two basic, three neutral, and four acidic compounds. HPLC and CE methods were developed to determine the quality of SGMX. Both methods were applied to determine the constituent amounts in a SGMX sample, and the results were statistically analyzed using validation parameters. The suitability of these two methods is compared and discussed.

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2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson (NJ, USA). The formic acid was analytical grade (Sigma, MO, USA). Purified, ultra-filtered water (Sinhan, Korea) was used. Marker compounds (5-hydroxymethylfuraldehhyde, geniposidic acid, chlorogenic acid, paeoniflorin, 20-hydroxyecdysone, coptisine, berberine, luteolin, glycyrrhizic acid) were isolated from herbs in the Pharmacognosy Laboratory at the Pharmacy School, Chungnam National University. Molecular structures were confirmed by comparing spectroscopic analyses with published data. The purities of marker compounds determined using HPLC were higher than 98%.

2.2. Preparation of sample and standard solutions

SGMX is an extract from a mixture of 14 herbs, *Rehmannia glutinosa* (Rehmanniae Radix Preparata), *Eucommia ulmoides* (Eucommiae Cortex), *Achyranthes japonica* (Achyranthis Radix), *Lycium chinese* (Lycii Fructus), *Poria cocos* (Hoelen Alba), *Paeonia lactiflora* (Paeoniae Radix), *Aconitum carmichaeli* (Aconiti Tuber), *Aralia continentalis* (Araliae Continentalis Radix), *Glycyrrhiza uralensis* (Glycyrrhizae Radix), *Cinnamomum cassia* (Cinnamoni Ramulus), *Angelica gigas* (Angelicae Radix), *Coptis japonica* (Coptidis Rhizoma), *Phyllostachys nigra* (Bambusae Caulis in Taeniam), and *Zingiber officinale* (Zingiberis Rhizoma). To prepare the mixture, the 14 crude herbs were combined and powdered, and the powdered mixture was immersed in 100 mL of water and boiled for 60 min; this procedure was repeated twice. The extracts were combined, concentrated to approximately 100 mL, and freeze-dried using a commercial freeze drier. All extracts were stored in sealed bottles at 4 °C prior to use. A stock solution of SGMX was prepared by dissolving 200 mg in 10 mL of water and filtering through a membrane filter. The reference marker compounds were accurately weighed and dissolved in methanol at concentrations of 2 mg/mL. Working standard solutions were prepared from the stock solutions by dilution with an appropriate volume of methanol. Quality control standard in high concentration was prepared by mixing the stock solutions to 300 mg/L for standards 1, 2 and 4, to 40 mg/mL for 3, 6 and 7, and 10 mg/mL for 5, 8 and 9, and diluted two and four times to prepare quality control standards in medium and low concentrations, respectively. These solutions were stored at 4 °C. All solutions were filtered through a 0.45-μm filter before analysis.

2.3. HPLC analysis

HPLC analyses were performed using a Shimadzu LC-10AD series HPLC system (Japan) with a column oven and a diode array detector (DAD). An Eclipse-DB-C_{18} column (2.1 mm × 150 mm, 5 μm, Agilent, Korea) was used at 25 °C for separation. The mobile phase consisted of 2% acetonitrile in water containing 0.05% formic acid (A) and 90% acetonitrile (B). Samples were eluted at a flow rate of 0.4 mL/min. A gradient elution was used according to the following schedule: 0% B for the first 5 min, a linear increase to 40% B from 5 to 40 min, a linear increase to 60% B from 40 to 50 min, and a linear decrease to 0% B from 50 to 60 min. The total analysis time was 70 min. UV spectra were recorded from 190 to 400 nm, and the monitoring wavelength was set at 250 nm. HPLC–MS analyses were carried out using a Shimadzu LC-MS-2010-EV linked simultaneously to an electrospray ionization (ESI) source operating in both negative and positive mode. LC–MS solution software was used to control the instruments for data acquisition and processing. The LC–MS was operated with a nebulizing gas flow rate of 1.4 L/min, CDL temperature of 250 °C, heat block temperature of 200 °C, detector voltage of 1.50 kV, and a CDL voltage of 15.0 V.

2.4. CE analysis

CE analyses were conducted using a HP^{3D}CE (Hewlett Packard, Germany) equipped with a DAD detector set at 230 nm. Instrument control and data acquisition were performed using HP^{3D}CE ChemStation software and an untreated fused-silica capillary (50 μm I.D. × 60 cm; 52 cm effective length, BGB Analytic, Germany). The sample was injected at a pressure of 50 mbar for 5 s with a constant applied voltage of 25 kV and a column temperature of 25 °C. The electrolyte was a buffer solution of 70 mM $Na_2B_4O_7$ and 10% methanol.

2.5. Method validation

Linearity was examined with standard solutions in 5 different concentrations. Linearity was evaluated by plotting the integrated peak area for each component against its corresponding solution concentration. Intra-day precision and accuracy were evaluated by analyzing quality control standards five times, and the analyses were performed by one operator in a single day. Inter-day variability was assessed by repeating quality control standard analyses on five consecutive days. Precision was expressed as the intra-day and inter-day percentage relative standard deviations. Reproducibility, expressed as the RSD, was calculated based on the retention and migration times over five replicate injections. Stability was determined by analyzing the standard stock solutions that had been stored for 1 week at room temperature or for 1 month at 4 °C.

3. Results and discussion

3.1. Optimization of HPLC method

Preliminary experiments attempted to separate the nine marker compounds selected for the quality control of SGMX (Fig. 1). Isocratic elution failed to separate the marker compounds, while a gradient elution (see Section 2.3) yielded a baseline resolution for all nine components. Among several acidic modifiers that were evaluated for the separation of acidic components in SGMX, formic acid showed the best separation capability with less peak tailing than with acetic or phosphoric acid. Fig. 2 shows a HPLC-DAD chromatogram of the marker mixture and SGMX using optimized HPLC conditions. All major components in SGMX and the marker compounds were baseline-resolved. For LC–MS analysis, the effects of CDL voltage, CDL temperature and nebulizing gas flow rate were examined in both positive and negative ion modes. Besides protonated ions, sodium/potassium adduct ions or ions with a neutral loss of H_2O were yielded in positive ion mode for all marker compounds. In negative ion mode, quasi-molecular ions $[M-H]^-$ and adduct ions of formate anion were observed for marker compounds 2, 3, 4 and 8. Table 1 summarizes the mass spectra data. The nine marker components in SGMX were identified by comparing their MS data, HPLC retention times, and UV spectra with those of reference standards.

3.2. Optimization of CE method

Phosphate and borate buffers (50–100 mM) were tested for their use as running buffers, and the borate buffer showed higher separation efficiency. Improved resolution was observed with higher buffer concentrations, especially for peaks coptisine (6) and berberine (7). However, higher buffer concentrations required longer separation times for each run. Based on the resolution of peaks 6 and 7 and the running time, a 70 mM borate buffer was selected.

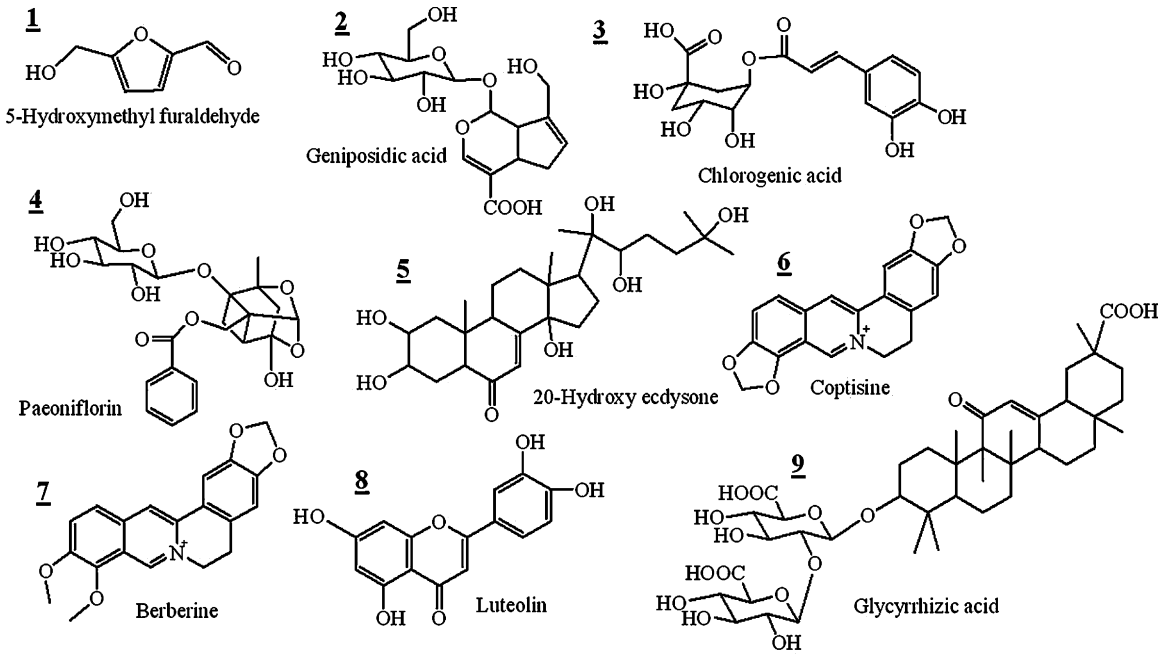

Fig. 1. Chemical structures of selected marker components in Samgiumgagambang (SGMX).

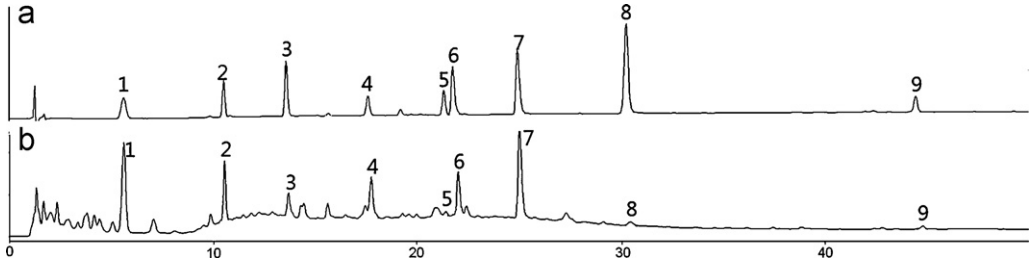

Fig. 2. HPLC-DAD chromatograms of (a) the marker compound mixture and (b) SGMX separated on an Eclipse-DB-C18 (2.1 mm × 150 mm) with mobile phase A. 2% acetonitrile (0.05% formic acid), B. 90% acetonitrile with gradient program of 0–5 min, 0% B; 40 min, 40% B; 50 min, 60% B. Peaks (1) 5-hydroxymethylfuraldehyde, (2) geniposidic acid, (3) chlorogenic acid, (4) paeoniflorin, (5) 20-hydroxyecdysone, (6) coptisine, (7) berberine, (8) luteolin, and (9) glycyrrhizic acid.

However, 20-hydroxyecdysone (5) could not be separated from the electro-osmotic flow. The resolution was pH-dependent; thus, borate buffer (70 mM) was investigated at various pH values, and pH 9.5 was the optimum for the separation of marker components from SGMX. Adding modifiers, such as sodium dodecyl sulfate (SDS) and sodium deoxycholate, reduced the column efficiency and did not improve the separation, but adding 10% methanol did improve the separation. The applied voltage and capillary temperature were optimized at 25 kV and 25 °C, respectively. Fig. 3 shows a representative eletropherogram of the marker components and SGMX using optimized CE conditions.

3.3. Linearity, precision, accuracy and reproducibility

Calibration curves were generated by plotting the chromatographic peak area as a function of concentration (mg/L) for each marker components from HPLC and CE data. The linear equation, calibration range, limit of detection, and limit of quantification are summarized in Table 2. The accuracy and precision were tested with the quality control standards in high, medium and high concentrations, and Table 3 shows the data acquired using a quality control standard in medium concentration of the nine marker compounds. The intra-day and inter-day precisions were less than 2.9%

Table 1
HPLC–MS identification of peaks obtained from the SGMX extracts in positive and negative ion mode.

Peak	t_R (min)	Positive ion mode	Negative ion mode	M.W.	Identification
1	5.7	127 $[M+H]^+$	–	126	5-Hydroxymethylfuraldehyde
2	11.4	443 $[M+HCOOH+Na]^+$, 397 $[M+Na]^+$	373 $[M-H]^-$, 419 $[M+HCOO]^-$	374	Geniposidic acid
3	14.0	355 $[M+H]^+$	353 $[M-H]^-$	354	Chlorogenic acid
4	18.2	519 $[M+K]^+$, 503 $[M+Na]^+$	479 $[M-H]^-$, 525 $[M+HCOO]^-$	480	Paeoniflorin
5	21.5	445 $[M-2H_2O+H]^+$	–	480	20-Hydroxyecdysone
6	22.8	338 $[M+H_2O]^+$, 320 M^+	–	320	Coptisine
7	25.9	336 M^+	–	336	Berberine
8	30.4	287 $[M+H]^+$	285 $[M-H]^-$	286	Luteolin
9	44.8	471 $[M-2G^a+H]^+$, 453 $[M-2G^a-OH]^+$	–	822	Glycyrrhizic acid

[a] Glucuronic acid.

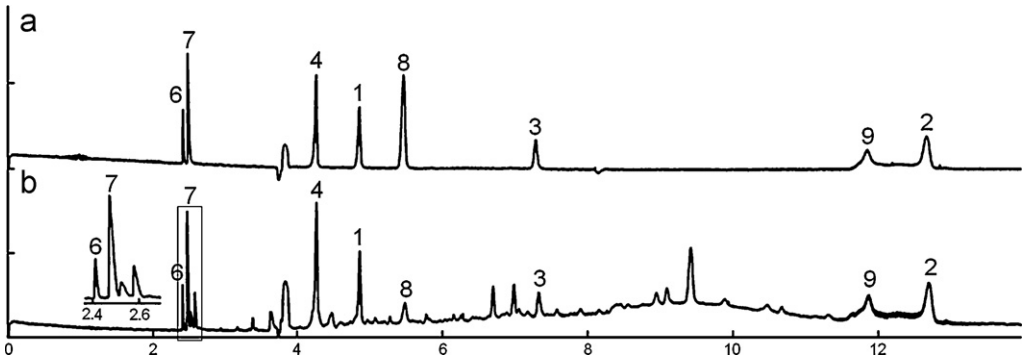

Fig. 3. Eletropherogram of (a) the marker compound mixture and (b) SGMX by capillary electrophoresis. Buffer: 70 mM borate (pH 9.5) containing 10% methanol. Peaks (1) 5-hydroxymethylfuraldehyde, (2) geniposidic acid, (3) chlorogenic acid, (4) paeoniflorin, (6) coptisine, (7) berberine, (8) luteolin, (9) glycyrrhizic acid.

Table 2
Calibration data for the nine marker compounds.

No. analytes	HPLC					CE				
	Calibration curve	r^2	Range[a]	LOD[b]	LOQ[c]	Calibration curve	r^2	Range[a]	LOD[b]	LOQ[c]
1	$y = 303x + 1.1$	0.9998	25–200	2	10	$y = 948x + 0.4$	0.9995	25–100	10	25
2	$y = 139x + 0.7$	0.9999	50–250	10	50	$y = 544x + 1.3$	0.9992	100–500	20	100
3	$y = 942x + 0.5$	0.9998	5–25	1	5	$y = 568x$	0.9995	10–50	2	10
4	$y = 152x + 0.8$	1.0000	50–200	10	50	$y = 1177x - 0.1$	0.9999	50–200	25	50
5	$y = 749x + 1.1$	0.9995	4–50	1	2	–	–	–	–	–
6	$y = 648x + 4.7$	0.9998	10–50	2	10	$y = 706x$	0.9995	20–100	5	20
7	$y = 1632x + 6.8$	0.9998	5–25	2	5	$y = 2281x - 0.6$	0.9996	10–50	2	10
8	$y = 1358x + 0.1$	0.9992	1–25	0.5	1	$y = 1905x$	0.9995	10–50	2	5
9	$y = 234x + 0.5$	0.9999	20–100	10	20	$y = 1960x$	0.9994	50–250	20	50

[a] Calibration range (mg/L).
[b] Limit of detection (mg/L).
[c] Limit of quantification (mg/L). No. analytes (1) 5-hydroxymethylfuraldehyde, (2) geniposidic acid, (3) chlorogenic acid, (4) paeoniflorin, (5) 20-hydroxyecdysone, (6) coptisine, (7) berberine, (8) luteolin, and (9) glycyrrhizic acid.

Table 3
Intra- and inter-day precision and accuracy of marker compound analyses.

No. analyte	QC[a]	HPLC ($n = 5$)				CE ($n = 5$)			
		Precision (%)		Accuracy (%)		Precision (%)		Accuracy (%)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1	150	0.9	1.8	99.4	100.1	2.2	2.9	98.9	103.5
2	150	0.8	2.7	100.6	104.3	2.3	3.5	105.5	108.6
3	20	0.9	1.6	98.8	99.7	2.5	3.4	99.5	101.3
4	150	1.1	3.4	98.6	98.9	2.1	3.9	101.5	102.6
5	5	0.8	2.5	99.9	101.7	–	–	–	–
6	20	2.4	3.5	100.8	103.1	1.8	3.0	99.6	100.3
7	20	2.9	4.7	100.1	100.7	1.9	2.7	100.2	100.9
8	5	2.6	3.8	99.7	100.2	1.5	2.9	101.2	99.1
9	5	2.9	4.5	100.8	102.9	2.5	4.1	104.2	106.1

[a] Quality control standard in medium concentration (mg/L). No. analytes (1) 5-hydroxymethylfuraldehyde, (2) geniposidic acid, (3) chlorogenic acid, (4) paeoniflorin, (5) 20-hydroxyecdysone, (6) coptisine, (7) berberine, (8) luteolin, and (9) glycyrrhizic acid.

Table 4
Concentrations (mg/g extract) of marker components in three SGMX batches.

Analyte	HPLC	CE
Geniposidic acid	11.20 ± 0.44	11.02 ± 0.56
5-Hydroxymethylfuraldehyde	6.33 ± 0.42	6.74 ± 0.75
Paeoniflorin	5.57 ± 0.03	5.86 ± 0.10
Coptisine	1.12 ± 0.04	1.02 ± 0.07
Berberine	1.07 ± 0.05	1.28 ± 0.02
Chlorogenic acid	0.50 ± 0.02	0.55 ± 0.05
20-Hydroxyecdysone	0.21 ± 0.01	–
Glycyrrhizic acid	0.21 ± 0.04	0.19 ± 0.05
Luteolin	0.05 ± 0.01	0.06 ± 0.02

and 4.7% for HPLC and 2.5% and 4.1% for CE, respectively, indicating good repeatability. The accuracy of the method ranged from 98.6 to 103.1% for HPLC and 98.9 to 108.6% for CE. All marker compounds were stable for at least 1 week at room temperature and for 1 month at 4 °C.

3.4. Application

HPLC and CE methods were tested to determine the nine marker components from three different batches of SGMX under optimized conditions. The contents of the components in SGMX determined using the two methods were quite similar (Table 4). All p-values of t-test and Mann–Whitney test for the statistical comparison of analytical results in HPLC and CE were higher than 0.05. These data indicated that both methods did not display any signifi-

cant differences and were suitable for routine quality control of SGMX.

4. Conclusions

Two methods, using HPLC and CE, were developed to determine nine marker components in SGMX and showed a good linear relationship, reproducibility, precision, and accuracy. The marker components in SGMX consisted of five dominant species, 5-hydroxymethylfuraldehyde, geniposidic acid, paeoniflorin, coptisine, and berberine, and four minor species, chlorogenic acid, 20-hydroxyecdysone, luteolin, and glycyrrhizic acid. For effective routine quality control of SGMX, determination of the five major components is recommended. There was no significant difference between the HPLC and CE methods based on the results for five major components in SGMX. However, paeoniflorin (4) was only partially separated from the adjacent component using HPLC, whereas these peaks were fully separated using CE. The component 20-hydroxyecdysone (5) was not separated from EOF in CE. The analysis times for one sample using HPLC and CE were 50 min and 14 min, respectively, indicating that the CE method has the advantages of shorter running time and higher separation efficiency.

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