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Quality Evaluation of Modified Bo-Yang-Hwan-O-Tang by Capillary Electrophoresis and High-performance Liquid Chromatography

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High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were used to identify five active components in the modified herbal decoction Bo-Yang-Hwan-O-Tang (mBHT), i.e., amygdalin, decursin, paeoniflorin, salvianolic acid B, and calycosin-7-*O*-β-D-glycoside. These components were identified by comparing their retention times and mass spectra with those of reference compounds. The conditions of both analytical methods were optimized and validated. Sufficient separation of target analytes was achieved using a buffer consisting of 40 mM sodium borate and 60 mM sodium dodecylsulfate (SDS) containing 10% methanol (pH 9.5) at 250 nm for CE analysis and gradient elution with a water-methanol mobile phase and ultraviolet (UV) photodiode array detector (DAD) at 250 nm for HPLC analysis. The mBHT components were determined within 65 min by HPLC and 16 min by CE. All calibration curves showed high linearity ($R > 0.999$) within the ranges tested. Intra-day and inter-day precision were less than 1.6% and 1.8% for HPLC and 2.5% and 4.8% for CE, respectively. The accuracy of the methods ranged from 98.8% to 102.3% for HPLC and from 95.9% to 108.2% for CE.

Key Words : Quality control, Modified Bo-Yang-Hwan-O-Tang, High-performance liquid chromatography, Capillary electrophoresis, Marker component

Introduction

Traditional herbal formulae contain several crude drugs at an intrinsic mass ratio to achieve beneficial bioactivities for clinical indications. Herbal products are generally believed to be effective and have low toxicity due to their performance in clinical applications and natural origin.[1-3] However, quality control and clarification of the therapeutic mechanisms of herbal prescriptions are bottlenecks hindering their development because these complex systems contain various chemical constituents.[4,5] The development of a quality-control methods for traditional herbal formulae typically requires identification and quantification of several characteristic components, which often requires 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).[6,7] Among these, HPLC is the most commonly used method. Liquid chromatography coupled with a photodiode array detector (DAD) and mass spectrometer (MS) is a powerful analytical tool for analysis of both known and unknown compounds in complex mixtures, making this technique ideal for herbal drug analysis.[8,9] Recently, CE has been recognized as an important alternative or complementary tool.[10,11] CE has not been used frequently for the analysis of multiple herbal preparations. However, CE is an economical technique and has many advantages that compensate for the drawbacks of HPLC, such as small sample volumes, small solvent consumption, and rapid separation with high efficiency.[12-14]

Bo-Yang-Hwan-O-Tang (BHT) is a decoction of seven herbs used in traditional Chinese herbal medicine for the treatment of cerebral or cardiac stroke and vascular dementia. Recently, BHT was modified (mBHT) by adding five herbs to BHT. mBHT has been shown to have biological activity toward ailments such as stroke, senility, vascular dementia, and heart damage, as well as thrombosis and immunomodulatory effects.[15,16] To standardize the pharmacological action of mBHT, an effective method of quality control is required.

The present study was performed to optimize CE and HPLC analytical methods for evaluation of the five major active compounds in mBHT. Here, we present the development, validation, and comparison of HPLC and CE methods for quality control of mBHT. The suitability of both methods is compared and discussed.

Materials and Methods

Chemicals and Reagents. HPLC-grade methanol was purchased from Burdick & Jackson (Morristown, NJ, USA). Formic acid was of analytical grade (Sigma, St. Louis, MO, USA). Sodium Phosphate and sodium borate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecylsulfate (SDS), sodium cholate (SC), and β-cyclo-

dextrin (β-CD) were from Wacker-Chemie GmbH (Munich, Germany). Water was purified using an ultrafiltration system (Shinhan Scientific, Seoul, Korea). Amygdalin standard was purchased from Sigma. Decursin, paeoniflorin, salvianolic acid B, and calycosin-7-*O*-β-D-glycoside were isolated from herbs in the Pharmacognosy Laboratory, School of Pharmacy, Chungnam National University, Daejeon, South Korea, and their structures were confirmed by comparing the results of spectroscopic analyses with published data. The purities of marker compounds determined using HPLC were greater than 98%.

Preparation of Sample and Standard Solutions. mBHT, an extract from a mixture of 12 crude drugs (Astragali Radix, Salviae Miltiorrhizae Radix, Angelicae Gigantis Radix, Paeoniae Radix Rubra, Achyranthis Radix, Lumbricus, Cnidii Rhizoma, Persicae Semen, Carthami Flos, Cinnamomi Ramulus, Polygalae Radix, and Acori Graminei Rhizoma) was prepared as follows: the 12 crude drugs were combined and powdered, and the mixture was immersed in 100 mL of boiling water for 3 h, filtered through a two-layer mesh, concentrated under vacuum at 700 mmHg for 15 h, and freeze-dried to yield 34.8% mBHT. The lyophilized mBHT powder was stored at 4 °C. A stock solution of mBHT was prepared by dissolving 200 mg in 10 mL of water and filtering through a membrane filter. Reference compounds were accurately weighed and dissolved in methanol at a concentration of 1 mg/mL. Working standard solutions were prepared from stock solutions by further dilution with an appropriate volume of methanol. Quality control standards were prepared in high concentration by mixing the stock solutions to 40 mg/L for standard (1), 200 mg/L for (2), 20 mg/L for (3), 300 mg/L for (4), and 100 mg/L for (5) and diluting two and four times to prepare quality control standards at medium and low concentrations, respectively. These solutions were stored at 4 °C. All solutions were filtered through a 0.45-μm filter before HPLC analysis.

HPLC Analysis. Analysis of mBHT was carried out using a Shimadzu LC-10AD series HPLC system (Shimadzu, Kyoto, Japan) with a column oven and a DAD. A C_{18} column (2.1 × 150 mm, 5 mm; Phenomenex, Torrance, CA) was used for separation at a column temperature of 25 °C. The mobile phase consisted of 10% methanol in water containing 0.05% formic acid (A) and 90% methanol (B) with a flow rate of 0.4 mL/min. A gradient program was used as follows: 0-30 min, linear increase from 0% to 40% B; 30-60 min, linear increase to 75% B and maintained at that level for a further 5 min; 65-70 min, linear decrease to 0% B. The total analysis time was 70 min. Ultraviolet (UV) spectra were recorded from 190 to 400 nm, and the monitoring wavelength was 250 nm. HPLC-MS analyses were carried out on a Shimadzu LCMS-2010-EV linked to an electrospray ionization (ESI) source operating in both negative- and positive-ion mode. LC-MS solution software was used to control the instruments for data acquisition and processing. The LC-MS was operated at 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 CDL voltage of 15.0 V.

CE Analysis. CE analyses were conducted using an $HP^{3D}CE$ (Hewlett Packard, Böblingen, Germany) equipped with a DAD detector at 250 nm. Instrument control and data acquisition were performed using $HP^{3D}CE$ ChemStation software and an untreated fused-silica capillary (BGB Analytik Vertrieb, Schlossböckelheim, Germany; 50 μm I.D. × 50 cm; 42 cm effective length). The analytical conditions were as follows: 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. In capillary zone electrophoresis (CZE), the electrolyte was a buffer solution consisting of 40 mM sodium borate containing 10% methanol adjusted to pH 9.5 with ammonia solution. In modified CZE, the electrolyte was a buffer solution of 40 mM sodium borate and 60 mM SDS containing 10% methanol adjusted to pH 9.5 with ammonia solution.

Method Validation. 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 in triplicate, performed by one operator in a single day. Inter-day variability was assessed by repeating quality control standard analysis on three consecutive days. Precision was expressed as the intra-day and inter-day percentage relative standard deviations (RSD). 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.

Results and Discussion

Optimization of HPLC Conditions. Five marker compounds were selected for quality control of mBHT (Fig. 1), and their separation was attempted in preliminary experiments. However, isocratic elution failed to separate the marker compounds. Gradient elution with a mobile phase consisting of 10% methanol containing 0.05% formic acid and 90% methanol yielded baseline resolution for all five components. Three acids (phosphoric, acetic, and formic) were used to modify the acidity of the mobile phase, and formic acid showed the best separation because it prevented peak tailing. Fig. 2 shows an HPLC-DAD chromatogram of a mixture of marker compounds and mBHT solution using optimized HPLC conditions. All major components in mBHT and the marker compounds were baseline-resolved. Identification and further characterization of the separated compounds in mBHT were performed by HPLC-MS in both negative- and positive-ion mode to obtain molecular ions and fragments. Table 1 presents a summary of the MS data. The five marker compounds in mBHT were identified by comparing their MS data, HPLC retention times, and UV spectra with those of reference standards.

Analytical Conditions for CE. CE was developed first in CZE mode with sodium phosphate and sodium borate buffers

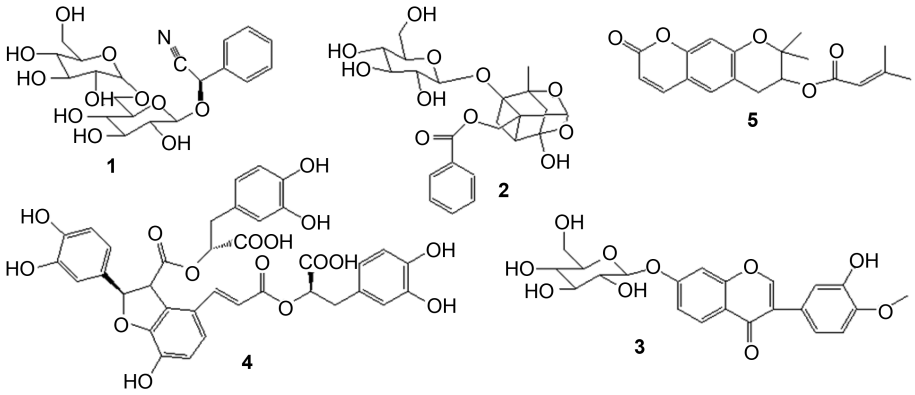

Figure 1. Chemical structures of selected marker compounds in mBHT.

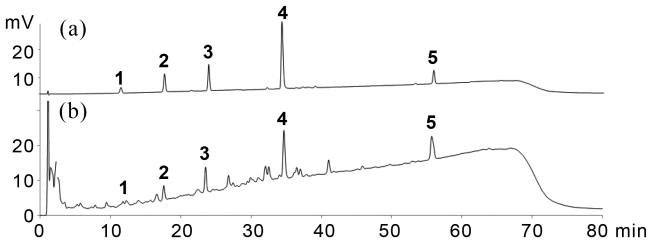

Figure 2. HPLC-DAD chromatogram of (a) standard mixture and (b) mBHT. Column: C_{18} (2.1 × 150 mm; Phenomenex); mobile phase A: 10% methanol (0.05% formic acid); B: 90% methanol; gradient program: 0-30 min, 40% B; 60 min, 75% B; 65 min, 75% B; flow rate: 0.4 mL/min; detection: 250 nm. Peaks (**1**) amygdalin, (**2**) paeoniflorin, (**3**) calycosin-7-*O*-β-D-glycoside, (**4**) salvianolic acid B, and (**5**) decursin.

in various concentrations (20-80 mM) as electrolytes. Sodium borate provided higher separation efficiency than sodium phosphate. After a series of trials, a buffer containing 40 mM sodium borate (pH 9.5) was able to separate the five marker compounds from mBHT samples. Paeoniflorin (**2**) and decursin (**5**) migrated together with electroosmotic flow (EOF) and an unknown compound, respectively, because they were neutral compounds. Thus, a modified CZE method was used to separate the marker compounds by introducing additives to the borate solution to form micelles or complexes, such as SDS, SC, and β-CD. Only SDS significantly improved resolution. Electrolytes containing 40 mM sodium borate (pH 9.5) and various concentrations of SDS were used to study the separation of the five marker compounds. When SDS was absent or at a concentration less than 40 mM, paeoniflorin (**2**) and decursin (**5**) migrated together with EOF and an unknown compound, respectively. With an SDS concentration of 60 mM, paeoniflorin (**2**) and decursin (**5**) were separated completely (Fig. 3). The applied voltage and capillary temperature were optimized at 25 kV and 25 °C, respectively.

Linearity, Precision, Accuracy and Reproducibility. Calibration curves were generated by plotting the chromatographic peak area as a function of extract concentration (mg/L) for each marker compound from HPLC and CE data. The linear equation, calibration range, limit of detection (LOD), and limit of quantification are summarized in Table 2. Table 3 shows precision and accuracy data acquired using a quality control standard containing known amounts of each of the five marker compounds. The component concentrations in the quality control standard were determined using the concentration of each marker compound based on

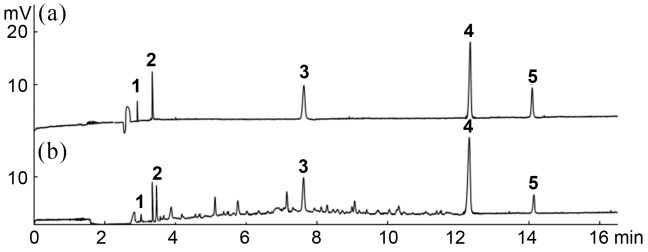

Figure 3. Electropherogram of (a) the marker compound mixture and (b) mBHT by modified CZE. Buffer: 40 mM borate, 60 mM SDS (pH 9.5) containing 10% methanol. Peaks (**1**) amygdalin, (**2**) paeoniflorin, (**3**) calycosin-7-*O*-β-D-glycoside, (**4**) salvianolic acid B, and (**5**) decursin.

Table 1. HPLC-MS identification of peaks from the mBHT extracts in positive and negative ion mode

Peak	t_R (min)	Identified *m/z* in		M.W.	Identification
		Positive ion mode	Negative ion mode		
1	11.2	496[M+K]$^+$	456[M-H]$^-$	457	Amygdalin
2	17.9	503[M+Na]$^+$, 521[M+H_2O+Na]$^+$	525[M+HCOO]$^-$, 479[M-H]$^-$	480	Paeoniflorin
3	24.4	501[M+3H_2O+H]$^+$, 447[M+H]$^+$	–	446	Calycosin-7-*O*-β-D-glycoside
4	35.8	741[M+Na]$^+$, 795[M+3H_2O+Na]$^+$	717[M-H]$^-$	718	Salvianolic acid B
5	56.4	329[M+H]$^+$	327[M-H]$^-$	328	Decursin

Table 2. Calibration data for five marker compounds

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 = 212x + 1.2	0.9996	20-100	2	10	y = 130x – 0.05	0.9998	20-100	10	20
2	y = 237x – 0.3	0.9998	10-200	5	25	y = 170x – 0.8	1.0000	25-200	20	100
3	y = 1300x + 0.4	0.9996	10-250	5	20	y = 6537x – 8.4	0.9997	10-50	10	25
4	y = 650x – 1.4	1.0000	50-200	1	5	y = 650x + 0.4	1.0000	50-200	15	50
5	y = 1145x – 2.6	1.0000	15-100	2	10	y = 2458x + 0.8	0.9995	20-200	20	50

[a]Calibration range (mg/L). [b]Limit of detection (mg/L). [c]Limit of quantification (mg/L). Analytes (**1**) amydalin, (**2**) paeoniflorin, (**3**) calycosin-7-*O*-β-D-glycoside, (**4**) salvianolic acid B, and (**5**) decursin.

Table 3. Precision and accuracy of marker compound analyses

Analytes	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	40	1.6	1.8	102.3	101.5	2.2	2.8	108.2	101.5
2	50	0.7	0.6	100.2	100.4	2.3	3.5	96.3	95.9
3	60	0.9	1.2	98.8	99.7	2.5	3.4	99.5	101.3
4	80	0.6	0.5	101.6	100.8	2.0	4.8	101.5	102.6
5	50	0.8	1.5	99.9	101.7	2.1	3.1	97.8	102.3

[a]Quality control standard in medium concentration (mg/L). Analytes (**1**) amydalin, (**2**) paeoniflorin, (**3**) calycosin-7-*O*-β-D-glycoside, (**4**) salvianolic acid B, and (**5**) decursin.

Table 4. Concentration (mg/g extract) of the five marker compounds in mBHT extracts analyzed by HPLC and CE

Analyte	HPLC	CE
Salvianolic Acid B	3.8±0.1	3.7±0.8
Calycosin-7-*O*-β-D-glycoside	3.2±0.2	3.4±0.5
Decursin	2.6±0.6	2.8±0.2
Paeoniflorin	2.5±0.5	2.4±0.3
Amygdalin 5	1.8±0.2	1.7±0.6

preliminary experiments with mBHT samples. The intra-day and inter-day precision were less than 1.6% and 1.8% for HPLC and 2.5% and 4.8% for CE, respectively, indicating good repeatability. The accuracy of the method ranged from 98.8% to 102.3% for HPLC and 95.9% to 108.2% for CE. Reproducibilities calculated based on the retention and migration times over five replicate injections were ranged from 0.4 to 1.2 for HPLC and 0.6 to 1.3 for CE. All marker compounds were stable for at least 1 week at room temperature and for 1 month at 4 °C.

Application. HPLC and CE were used to identify five compounds in mBHT under selected conditions. With respect to precision, accuracy, and LOD, HPLC showed slightly better results than CE. However, CE had shorter analysis times than HPLC, indicating that CE could be complementary to HPLC. The contents of the five marker compounds found in mBHT using the two validated methods were similar (Table 4). Statistical analysis of HPLC and CE results using the *t* test and the Mann-Whitney test showed *P*-values greater than 0.05. These data indicated that the two methods did not differ significantly and are suitable for routine quality control of mBHT.

Conclusion

HPLC and CE were used to identify five components in mBHT by optimizing the pH, buffer composition, and buffer concentration. Each compound was identified by comparing retention times and mass spectra with those of reference compounds. Although both HPLC and CE showed good linear relationships between the peak-area ratio, reproducibility, precision, and accuracy, the LOD of CE was lower than that of HPLC. There was no significant difference between HPLC and CE based on the results for five major components in mBHT. However, amygdalin (**1**) was only partially separated from the adjacent component using HPLC, whereas these peaks were successfully separated using CE. These results could be used for scientific quality control of herbal medicines and contribute to the modernization of traditional medicines.

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