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Chromatographic fingerprints and quantitative analysis for the quality evaluation of *Bao-Yuan Decoction* by ultra-performance liquid chromatography coupled with diode array and evaporative light scattering detection

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ABSTRACT

Bao-Yuan Decoction (BYD), a widely used traditional Chinese medicine formula, is worth developing into modern dosage forms. To assess the quality of traditional decoction, the commonly used ultra-performance liquid chromatography coupled with diode array and evaporative light scattering detection (UPLC-DAD/ELSD) method was initially applied to develop the analytical methods for the qualitative fingerprints and simultaneous quantitation of multiple marker compounds in BYD. Based on 16 batches of BYD prepared from multiple batches of qualified crude herbs combined randomly, the characteristic fingerprints were generated, with 41 and 19 common peaks detected by DAD and ELSD, respectively. Furthermore, ginsenosides Re, Rg₁ and Rb₁, calycosin-7-glucoside, calycosin, liquiritin, isoliquiritin apioside, isoliquiritin, glycyrrhizic acid and cinnamic acid were qualified as marker compounds to represent the herbs composing the formula. The characteristic fingerprints and the content ranges of multiple batches of the decoction were obtained, thus providing guidance for the quality control of modern dosage forms. The combination of these qualitative and quantitative methods will be an effective operational measure by which to evaluate and control the quality of BYD from traditional decoction to modern dosage forms.

KEYWORDS

Bao-Yuan Decoction, fingerprints, quantitation, quality evaluation

INTRODUCTION

Bao-Yuan Decoction (BYD) is a well-known formula in traditional Chinese medicine (TCM) used for the treatment of *Qi* vacuity, and it is applied to treat coronary heart disease with chronic heart failure [1], renal anemia [2] and cellular immune deficiency [3] in modern clinical diagnoses. It is composed of Ginseng radix et rhizoma (*Renshen*), Astragali radix (*Huangqi*), Glycyrrhizae radix et rhizoma (*Gancao*), and Cinnamomi cortex (*Rougui*), which is a formula originally recorded in *Bo Ai Xin Jian*, basically fixed in *Jing Yue Quan Shu* during the Ming dynasty and widely used in TCM clinics for more than 400 years [4]. In consideration of its safety and effectiveness, it is valuable to develop into modern dosage forms, such as granules, instead of traditional decoction. Therefore, it is crucial to analyze the quality attributes of decoction prepared by the traditional procedure that could be applied to evaluate

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the quality of modern forms to ensure the consistency of clinical efficacy with the traditional formula.

An integrated strategy was developed by coupling ultra-performance liquid chromatography (UPLC), quadrupole time-of-flight (Q-TOF) with hybrid triple quadrupole-linear ion trap mass spectrometry (Qtrap-MS) and applied to profile the comprehensive chemical compounds in BYD. As many as 236 compounds were plausibly or unambiguously identified, and 175 compounds were quantified or relatively quantified [5]. This work was excellent, but the application of this strategy was limited because Q-TOF and Qtrap-MS have not been widely equipped in many pharmaceutical factories in China.

In the present study, the commonly equipped diode array detector (DAD) was initially applied for the quantitative fingerprint analysis, supplemented by an evaporative light-scattering detector (ELSD), and the rapid quantitation of multiple major marker compounds (see Fig. 1) of 16 batches of BYD prepared by the traditional procedure was conducted. These methods are more applicable for evaluating the quality of modern dosage forms during industrial production.

EXPERIMENTAL

Chemicals, reagents and materials

The reference standards of ginsenosides Re (Batch No. 110754-201626), Rg₁ (Batch No. 110703-201731) and Rb₁

(Batch No. 110704-201726), calycosin-7-glucoside (Batch No. 111920-201606), liquiritin (Batch No. 111610-201607), glycyrrhizic acid ammonium salt (Batch No. 110731-201619), and cinnamic acid (Batch No. 110786-201604) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Calycosin (Batch No. wkq17022102), isoliquiritin apioside (Batch No. wkq17012408), and isoliquiritin (Batch No. wkq17042112) were purchased from Weikeyi Biological Co., Ltd. (Chengdu, China). The purities were all above 98%.

Acetonitrile, methanol and ammonium acetate of HPLC grade were obtained from ANPEL Laboratory Technologies, Inc. (Shanghai, China). Deionized water was prepared using a Millipore Milli-Q water purification system (Millipore, MA, USA).

Fifteen batches of each kind of crude herbs, Ginseng radix et rhizoma, Astragali radix, Glycyrrhizae radix et rhizoma, and Cinnamomi cortex, were mainly supplied by Shineway Pharmaceutical Group Co., Ltd. and were purchased from a TCM market (Bozhou, Anhui, China). The collection information is listed in Supplementary Table 1. Their botanical origins were identified by the corresponding author, and voucher specimens were deposited at the School of Pharmacy, Shanghai Jiao Tong University. The crude herbs were examined according to the Chinese pharmacopoeia [6–9] and were all qualified. Then, the crude herbs were sliced for the decoction.

Waters Oasis HLB LP solid-phase extraction (SPE) cartridges (6 cc/500 mg, Part No. 18600015) were purchased

Fig. 1. Chemical structures of the marker compounds in BYD. Glc, glucose; Glc A, glucuronic acid; Rha, rhamnose; Api, apiose

from Waters Technology (Shanghai) Co., Ltd. (Shanghai, China).

Instrumentation

The analysis was performed on an Agilent 1290 UPLC system equipped with binary solvent delivery system, auto-sampler, column temperature controller and diode array detector (DAD) tandem evaporative light-scattering detector (ELSD). An Agilent Proshell 120 EC-C18 (100 mm × 4.6 mm, 2.7 µm) was employed during the experiment.

Chromatographic conditions

The column temperature was maintained at 30°C. The mobile phase was composed of acetonitrile (A) and ammonium acetate aqueous solutions at 1 mmol/L (B, pH 4.5), with a flow rate of 0.5 mL/min. Five µL aliquots of the sample solutions were injected for analysis.

The gradient elution for qualitative fingerprints was as follows: 0–5 min, 5% A; 5–6 min, 5%–7% A; 6–16 min, 7%–23.5% A; 16–24 min, 23.5% A; 24–26 min, 23.5%–26% A; 26–30 min, 26%–32% A; 30–37.5 min, 32%–52% A; 37.5–40 min, 52%–90% A; 40–44 min, 90% A; and 44–46 min, 90% A. The wavelength was set at 260 nm. The nitrogen flow rate and the atomization and vaporization temperatures were separately set at 1.6 L/min, 40 and 60 °C.

The gradient elution for the quantitation of multiple marker compounds was as follows: 0–10 min, 7%–23.5% A; 10–16 min, 23.5%; 16–18 min, 23.5%–26% A; 18–22 min, 26%–32% A; 22–27 min, 32%–52% A; and 27–32 min, 52%–90% A. The wavelengths were set at 203 nm for ginsenosides Re, Rg₁ and Rb₁; 260 nm for calycosin-7-glucoside, calycosin, liquiritin, glycyrrhizic acid and cinnamic acid; and 360 nm for isoliquiritin apioside and isoliquiritin.

Preparation of standard solutions

The stock solutions of standards were prepared as follows. First, 10 mg of ginsenoside Re, Rg₁ and Rb₁; 12.5 mg of calycosin-7-glucoside, calycosin, liquiritin, isoliquiritin apioside, glycyrrhizic acid ammonium salt and cinnamic acid; and 5 mg of isoliquiritin were accurately weighted and then dissolved in 25 mL of methanol, respectively. Five different concentrations in series of the standard solutions were prepared by mixing and diluting the stock solutions with methanol, including 400, 200, 100, 50 and 25 µg/mL of ginsenoside Re, Rg₁ and Rb₁; 500, 250, 50, 10 and 2 µg/mL of calycosin-7-glucoside, calycosin, liquiritin, isoliquiritin apioside, glycyrrhizic acid ammonium salt and cinnamic acid; and 200, 100, 20, 4 and 0.8 µg/mL of isoliquiritin. They were applied to establish the calibration curves. All the solutions were stored at 4°C.

Preparation of decoctions

The sliced Ginseng radix et rhizoma (3.75 g), Astragali radix (7.5 g), Glycyrrhizae radix et rhizoma (1.88 g), and Cinnamomi cortex (0.75 g) were mixed and soaked thoroughly in a 10-fold volume of water for 30 min, decocted for 40 min

with boiling, then filtered through double-layered gauze, which followed the traditional procedure. Decoctions of the individual herbs and formulas lacking individual herbs were prepared by the same procedure.

These decoctions of BYD (the complete formula), ginseng, lack-ginseng, astragali, lack-astragali, glycyrrhizae, lack-glycyrrhizae, cinnamomi, and lack-cinnamomi were all concentrated under reduced pressure to the same volume of 25 mL. These decoctions were applied to evaluate the specificity of the quantitative method.

Fifteen batches of BYD were made from the four kinds of herbs selected randomly from each of the 15 batches. Every batch of each individual herb was mixed in equal amount and then used to compose the complete formula. The combination information is listed in Table 1. Finally, 16 batches of BYD were analyzed to create the characteristic fingerprints and were quantified.

Preparation of sample solutions

One mL of the decoction prepared above with a 6-fold volume of ethanol was centrifuged at 12,000 rpm for 10 min after vortexing. The supernatant was dried under nitrogen, dissolved in 2 mL of methanol, and then filtered through a 0.22 µm membrane filter and analyzed for qualitative fingerprints and quantitation (except for ginsenosides Re, Rg₁ and Rb₁).

The other 5 mL of the decoction was loaded onto pre-conditioned SPE cartridges then eluted successively with 10 mL of water, and 40% and 60% methanol aqueous solutions.

Table 1. Combination information of BYD from the four kinds of herbs

Batch No. of BYD	Batch No. of each kind of herbs			
	Ginseng radix et rhizoma	Astragali radix	Glycyrrhizae radix et rhizoma	Cinnamomi cortex
S1	RS1709275	HQ1708143	GC1804193	RG170832
S2	RS180312	HQ1708146	GC180321	RG-1
S3	RS1709271	HQ-5	GC1804194	RG-5
S4	RS1709273	HQ-6	GC180314	RG-2
S5	RS-7	HQ-2	GC180312	RG180311
S6	RS-8	HQ180321	GC180324	RG170834
S7	RS180315	HQ1708144	GC180323	RG180313
S8	RS180322	HQ-3	GC180313	RG180313
S9	RS180313	HQ180323	GC180322	RG-3
S10	RS180324	HQ180322	GC180325	RG-4
S11	RS1709272	HQ1708142	GC180315	RG170831
S12	RS1709274	HQ1708145	GC1804191	RG180312
S13	RS180314	HQ-1	GC1804195	RG-6
S14	RS180323	HQ-4	GC1804192	RG180314
S15	RS180311	HQ180324	GC180311	RG170833
S16	Every batch above was mixed in equal amount.	Every batch above was mixed in equal amount.	Every batch above was mixed in equal amount.	Every batch above was mixed in equal amount.

The last effluent was concentrated to dryness under reduced pressure, dissolved in 5 mL of methanol, and then filtered through a 0.22 µm membrane filter and analyzed for the quantitation of ginsenosides Re, Rg₁ and Rb₁, specifically.

Method validation for the qualitative fingerprints of BYD by UPLC-DAD/ELSD

The validation of the qualitative method for chromatographic fingerprints included assessing the stability, precision and repeatability.

One sample solution of BYD (S1) was analyzed at room temperature every 2 h for 12 hours after the sample preparation for the sample stability, and it was injected 6 times for the instrumental precision. Six sample solutions of BYD prepared from the same batch of decoction in parallel were analyzed for the repeatability.

Peaks in sample solution with high responses, moderate retention times, and good stability and resolution were considered as references. The ratios of the peak areas and retention times over those of the reference in the same sample solution were calculated as the relative peak area (RPA) and relative retention time (RRT), the relative standard deviation (RSD) of which was calculated for each kind of chromatogram to evaluate the stability, precision and repeatability, respectively. Furthermore, the chromatographic similarities of each item were calculated by the software Similarity Evaluation System for Chromatographic Fingerprint of TCM, which will be illustrated in next section, and the results were also considered in the validation of this qualitative method.

Sample analysis for the qualitative fingerprints of BYD

Sample solutions prepared from 16 batches of BYD were analyzed by the qualitative method.

The DAD and ELSD chromatograms in AIA form were processed by the software Similarity Evaluation System for Chromatographic Fingerprint of TCM (version 2012), as recommended by the National Medical Products Administration and Chinese Pharmacopoeia Committee. Multi-point correction was applied to generate a chromatographic fingerprint, and peak matching and identification were conducted based on the chromatogram of S1, which was considered as a reference. Finally, the characteristic fingerprints were created by the median method, by referring to which the chromatographic similarities of 16 batches of BYD were evaluated.

Method validation for the quantitation of multiple marker compounds in BYD by UPLC-DAD

The validation of the quantitative method included the specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, repeatability, stability, and accuracy.

The specificity was evaluated by comparing the chromatograms of BYD, individual herbs and decoctions lacking individual herbs with those of the standard solutions.

The calibration curves were established using the linear least-square regression equation based on the known concentrations and corresponding peak areas of each marker compound in the standard solutions.

Standard solutions at the lowest concentration used for calibration were further diluted until the signal-to-noise ratio (S/N) of the analyte was 3 and 10, and then the corresponding concentrations were considered the LOD and LOQ, respectively.

The instrumental precision was validated by performing intra- and inter-day assays of the mixed standard solutions. Six replicate injections were analyzed in a single day for the intra-day precision assay, with injections lasting for three consecutive days for the inter-day precision assay. The repeatability was examined by analyzing six sample solutions of BYD prepared from the same batch of decoction in parallel. The sample stability was analyzed at room-temperature every 2 h for 12 hours after the sample preparation. The RSD of the peak area of each marker compound was calculated to evaluate the precision, repeatability and stability.

For the accuracy, the decoction of BYD, in which the contents of marker compounds were already known, was spiked with standards at fixed amounts of 80%, 100% and 120% of the original contents, respectively. Three replicates at each level were conducted according to the sample preparation. The average recovery (%) of the marker compound in sample solutions was calculated by the following equation:

$$\text{Recovery}(\%) = [(\text{Detected amount} - \text{Original amount}) / \text{Spiked amount}] \times 100\%$$

Sample analysis for the quantitation of multiple marker compounds in BYD

Sample solutions prepared from 16 batches of BYD were analyzed by the quantitative method. Then, the contents of marker compounds in the decoctions were calculated based on the established calibration curves.

RESULTS

Method validation for the qualitative fingerprints of BYD by UPLC-DAD/ELSD

The major compounds in the BYD were as follows. Tetracyclic triterpenoid saponins from Ginseng radix et rhizoma were absorbed around the terminal in the ultraviolet region; pentacyclic triterpenoid saponins from Glycyrrhizae radix et rhizoma responded highly around 260 nm; and flavonoids from Glycyrrhizae radix et rhizoma and Astragali radix responded highly around 260 and 360 nm. Comparing the responses at 203, 260 and 360 nm, 260 nm was considered the proper wavelength for the moderate response and the larger peak capacity.

Twenty-seven and 14 major peaks were selected in chromatograms of DAD and tandem ELSD, of which

glycyrrhizic acid was considered as the reference peak (detected at 31.32 min by DAD and 31.42 min by ELSD). The major results of the precision, repeatability, and stability are summarized in Table 2.

In the validation of precision, the RSDs of RPA and RRT were under 2.9% and 0.2% for DAD and under 3.6% and 0.1% for ELSD chromatograms. The similarities were all

above 0.999 for both DAD and ELSD chromatograms. In the validation of repeatability, the RSDs of RPA and RRT were under 3.1% and 1.1% for DAD and under 3.4% and 0.2% for ELSD chromatograms. The similarities were above 0.983 for DAD and above 0.999 for ELSD chromatograms. These results showed that the precision of the apparatus was sound and that the chromatographic condition was repeatable. In

Table 2. Method validation for the qualitative fingerprints of BYD by UPLC-DAD/ELSD

Detector	Peak No.	t_R^1 (min)	Precision ($n = 6$)				Repeatability/reproducibility ($n = 6$)				Stability (7 time points in 12 h)			
			Mean RRT ²	RSD (%)	Mean RPA ³	RSD (%)	Mean RRT	RSD (%)	Mean RPA	RSD (%)	Mean RRT	RSD (%)	Mean RPA	RSD (%)
DAD (260 nm)	1	3.71	0.12	0.2	0.29	0.3	0.12	1.1	0.30	0.9	0.12	0.3	0.29	0.2
	2	3.84	0.12	0.2	0.13	0.3	0.12	0.5	0.14	1.9	0.12	0.3	0.13	0.5
	3	4.45	0.14	0.2	0.08	0.4	0.14	0.5	0.08	1.0	0.14	0.3	0.08	1.5
	4	7.38	0.24	0.2	0.26	0.8	0.23	0.5	0.26	0.5	0.24	0.3	0.26	0.8
	5	11.85	0.38	0.1	0.01	0.5	0.38	0.2	0.01	1.4	0.38	0.3	0.01	1.6
	6	14.32	0.46	0.1	0.02	0.7	0.46	0.1	0.02	0.6	0.46	0.1	0.02	1.1
	7	14.65	0.47	0.1	0.01	0.6	0.47	0.1	0.01	3.1	0.47	0.1	0.01	2.7
	8	15.46	0.49	0.1	0.01	1.0	0.49	0.1	0.01	0.8	0.49	0.1	0.01	1.5
	9	15.57	0.50	0.1	0.03	0.3	0.50	1.3	0.03	1.2	0.50	0.1	0.03	0.7
	10	16.75	0.54	0.1	0.11	0.3	0.53	0.1	0.11	0.4	0.54	0.1	0.11	0.2
	11	17.22	0.55	0.1	0.57	0.1	0.55	0.1	0.57	0.2	0.55	0.1	0.57	0.2
	12	17.46	0.56	0.1	0.65	0.3	0.56	0.1	0.64	0.6	0.56	0.1	0.64	0.4
	13	17.75	0.57	0.1	0.29	2.9	0.57	0.1	0.28	0.8	0.57	0.1	0.28	0.8
	14	18.23	0.58	0.1	0.25	0.1	0.58	0.1	0.25	1.3	0.58	0.1	0.25	0.2
	15	18.59	0.59	0.1	0.02	1.3	0.59	0.1	0.02	1.4	0.59	0.1	0.02	3.1
	16	22.04	0.70	0.1	0.08	0.5	0.70	0.1	0.08	0.7	0.70	0.1	0.08	0.7
	17	22.52	0.72	0.1	0.05	0.3	0.72	0.1	0.05	0.6	0.72	0.1	0.05	0.9
	18	23.19	0.74	0.1	0.07	0.3	0.74	0.1	0.07	0.3	0.74	0.1	0.07	0.7
	19	23.72	0.76	0.1	0.10	0.7	0.76	0.1	0.11	0.4	0.76	0.1	0.10	0.9
	20	24.21	0.77	0.1	0.21	0.2	0.77	0.1	0.21	0.3	0.77	0.1	0.21	0.3
	21	27.90	0.89	0.1	0.06	2.1	0.89	0.1	0.07	0.8	0.89	0.1	0.07	2.7
	22	29.57	0.95	0.1	0.32	0.1	0.94	0.1	0.32	0.8	0.95	0.1	0.32	0.2
	23	30.99	0.95	0.1	0.08	0.1	0.99	0.1	0.08	0.5	0.99	0.1	0.08	0.4
	24*	31.32	1	0	1	0	1	0	1	0	1	0	1	0
ELSD	25	32.54	1.04	0.1	0.18	0.7	1.04	0.1	0.18	1.1	1.04	0.1	0.18	0.9
	26	36.99	1.18	0.1	0.15	0.1	1.18	0.1	0.15	0.2	1.18	0.1	0.15	0.2
	27	40.41	1.33	0.1	0.01	0.7	1.33	0.1	0.01	2.6	1.33	0.1	0.01	0.8
	1	17.27	0.55	0.1	0.17	2.1	0.55	0.1	0.17	1.5	0.55	0.1	0.17	2.7
	2	17.56	0.56	0.1	0.78	1.8	0.56	0.1	0.79	1.9	0.56	0.1	0.78	1.7
	3	17.85	0.57	0.1	0.20	2.4	0.57	0.1	0.21	2.1	0.57	0.1	0.20	2.3
	4	23.32	0.75	0.1	0.14	3.4	0.74	0.2	0.15	3.4	0.75	0.3	0.16	3.7
	5	23.58	0.75	0.1	0.07	3.0	0.75	0.2	0.07	2.5	0.75	0.3	0.06	1.7
	6	29.38	0.93	0.1	0.03	3.4	0.93	0.1	0.03	3.0	0.93	0.1	0.03	2.5
	7*	31.42	1	0	1	0	1	0	1	0	1	0	1	0
	8	33.81	1.08	0.1	0.03	3.3	1.08	0.1	0.03	1.4	1.08	0.1	0.03	1.7
	9	34.28	1.09	0.1	0.31	1.8	1.09	0.1	0.32	2.8	1.09	0.1	0.31	1.7
	10	34.68	1.10	0.1	0.13	1.9	1.10	0.1	0.14	2.5	1.10	0.1	0.14	1.1
	11	35.08	1.12	0.1	0.12	3.1	1.12	0.1	0.12	1.6	1.12	0.1	0.12	1.9
	12	36.05	1.15	0.1	0.04	2.1	1.15	0.1	0.04	1.8	1.15	0.1	0.04	1.7
	13	40.08	1.28	0.1	0.02	2.0	1.28	0.1	0.02	3.2	1.28	0.1	0.02	3.6
	14	40.56	1.29	0.1	0.02	3.6	1.29	0.1	0.02	2.7	1.29	0.1	0.02	3.5

¹ t_R , retention time.

²RRT, relative retention time.

³RPA, relative peak area.

*Reference peak.

addition, the detector of DAD was better than that of ELSD based on its higher peak capacity due to its lower detective limit.

In the validation of stability, the RSDs of RPA and RRT were under 3.1% and 0.3% for DAD and under 3.7% and 0.3% for ELSD chromatograms. The similarities were above 0.986 for DAD and above 0.999 for ELSD chromatograms. The results showed that the sample solution was stable within 12 h.

Sample analysis for the qualitative fingerprints of BYD

Based on 16 batches of BYD, the characteristic fingerprints detected by DAD and ELSD were separately created, in which 41 and 19 common peaks were labeled, respectively (see Fig. 2). ELSD is the common detector, but DAD only responds to chemicals with conjugated double bonds. Thus, the peak capacity of ELSD should be more than that of DAD. However, the LOD of ELSD is much higher than that of DAD. Therefore, the peak capacity of DAD was much more than that of tandem ELSD at the same low concentration.

Furthermore, peak attributions in the characteristic fingerprints were determined by comparison with chromatograms of the individual herb (see Tables 3 and 4). According to the results, 26, 2, 2 and 2 peaks were specifically attributed to *Glycyrrhizae radix et rhizoma*, *Astragali radix*, *Cinnamomi cortex* and *Ginseng radix et rhizoma*, respectively, all of which accounted for 78.05% of the total common peaks in the DAD chromatogram under 260 nm. In the ELSD chromatogram, 8, 5 and 3 peaks were specifically attributed to *Glycyrrhizae radix et rhizoma*, *Ginseng radix et rhizoma* and *Astragali radix*, all of which accounted for 84.21% of the total common peaks. No peak was attributed to *Cinnamomi cortex*, probably due to the low response to the low concentration. However, ELSD was a good supplement due to its good response to the compounds from *Ginseng radix et rhizoma*, which were mostly terpenoid saponins.

Compared to the characteristic fingerprints, the proportion of non-common peaks in S1 (the reference batch) was 15.93% for DAD and 11.61% for ELSD chromatograms. The similarities between the characteristic fingerprints and the chromatograms of 16 batches of BYD were not less than 0.888 as detected by DAD (see Table 8) and 0.862 as

Fig. 2. The characteristic fingerprints of BYD detected by DAD at 260 nm (A) and ELSD (B).

*, Reference peak. The attributions of the numbered common peaks were summarized in Tables 3 and 4

Table 3. Major parameters of 41 common peaks in the characteristic fingerprint of UPLC-DAD based upon 16 batches of BYD

Peak No.	t_R^1 (min)	RSD of RRT ² (%, $n = 16$)	RSD of RPA ³ (%, $n = 16$)	Attribution of herbs	Identification by standards
1	3.05	0.7	31.1	Ginseng radix et rhizoma, Astragali radix, Glycyrrhizae radix et rhizoma, Cinnamomi cortex	/
2	3.72	0.4	20.8	Ginseng radix et rhizoma, Astragali radix, Cinnamomi cortex	/
3	3.88	1.7	45.5	Ginseng radix et rhizoma, Astragali radix	/
4	4.46	0.6	55.9	Ginseng radix et rhizoma, Astragali radix	/
5	5.10	0.3	41.3	Glycyrrhizae radix et rhizoma	/
6	7.40	0.5	34.5	Ginseng radix et rhizoma, Astragali radix	/
7	9.07	0.3	25.7	Ginseng radix et rhizoma	/
8	9.56	0.2	49.7	Ginseng radix et rhizoma	/
9	11.03	0.1	22.1	Not attributed	/
10	11.19	0.3	58.9	Glycyrrhizae radix et rhizoma	/
11	11.85	0.2	44.1	Glycyrrhizae radix et rhizoma, Cinnamomi cortex	/
12	12.54	0.2	21.4	Glycyrrhizae radix et rhizoma	/
13	13.47	0.1	35.1	Glycyrrhizae radix et rhizoma	/
14	13.66	0.1	54.2	Glycyrrhizae radix et rhizoma	/
15	13.90	0.1	21.9	Cinnamomi cortex	/
16	14.31	0.1	35.2	Glycyrrhizae radix et rhizoma	/
17	14.64	0.1	30.4	Glycyrrhizae radix et rhizoma	/
18	15.44	0.1	52.5	Glycyrrhizae radix et rhizoma	/
19	15.56	0.1	16.7	Glycyrrhizae radix et rhizoma	/
20	16.70	0.6	50.5	Glycyrrhizae radix et rhizoma	/
21	17.20	0.1	32.1	Astragali radix	Calycosin-7-glucoside
22	17.45	0.1	39.9	Glycyrrhizae radix et rhizoma	/
23	17.75	0.1	47.9	Glycyrrhizae radix et rhizoma	Liquiritin
24	18.23	0.1	31.3	Cinnamomi cortex	Cinnamic acid
25	18.57	0.1	38.7	Glycyrrhizae radix et rhizoma	/
26	20.05	0.2	21.5	Glycyrrhizae radix et rhizoma	/
27	20.34	0.2	21.3	Glycyrrhizae radix et rhizoma	/
28	22.04	0.1	46.1	Glycyrrhizae radix et rhizoma	Isoliquiritin apioside
29	22.48	0.1	31.6	Glycyrrhizae radix et rhizoma	/
30	23.12	0.2	47.1	Glycyrrhizae radix et rhizoma	/
31	23.73	0.2	41.1	Glycyrrhizae radix et rhizoma	Isoliquiritin
32	24.16	0.1	46.6	Astragali radix, Glycyrrhizae radix et rhizoma	Ononin
33	27.9	0.1	32.1	Glycyrrhizae radix et rhizoma	/
34	29.18	0.2	21.8	Glycyrrhizae radix et rhizoma	/
35	29.57	0.1	48.6	Astragali radix	Calycosin
36	30.99	0.2	45.3	Glycyrrhizae radix et rhizoma	/
37*	31.29	0.3	40.6	Glycyrrhizae radix et rhizoma	Glycyrrhizic acid
38	32.54	0.2	48.9	Glycyrrhizae radix et rhizoma	/
39	36.92	0.3	36.2	Astragali radix, Glycyrrhizae radix et rhizoma	Formononetin
40	40.41	0.1	17.5	Glycyrrhizae radix et rhizoma	/
41	41.67	0.1	31.3	Glycyrrhizae radix et rhizoma	/

¹ t_R , retention time.²RRT, relative retention time.³RPA, relative peak area.

*Reference peak.

detected by ELSD. These results revealed that there were many different compounds (peaks) in the different batches of decoctions. In this study, crude herbs were collected from major or genuine producing areas, considering the supply and quality of the materials. The qualified herbs were sliced to prepare them for the decoction. Then, 15 batches of BYD (S1 to S15, see Table 1) were prepared from the four kinds of herbs selected randomly from each of the 15 batches. These procedures avoided subjective results and represented

diversity, based upon which the characteristic fingerprints were obtained as universal as possible. In the characteristic fingerprints, peak No. 37 (glycyrrhizic acid) detected by DAD and peak No. 13 (ginsenoside Rb₁) detected by ELSD were considered reference peaks, respectively. Both kinds of chromatograms showed that the RSDs of RRT of the 16 batches of BYD were very small (less than 1.7%), while the RSDs of RPA were extremely large (even reached 92.9% of Peak No. 17 detected by ELSD). This result also showed that

Table 4. Major parameters of 19 common peaks in the characteristic fingerprint of UPLC-ELSD based upon 16 batches of BYD

Peak No.	t_R^1 (min)	RSD of RRT ² (%, $n = 16$)	RSD of RPA ³ (%, $n = 16$)	Attribution of herbs	Identification by standards
1	3.16	0.32	20.74	Ginseng radix et rhizoma, Astragali radix	/
2	3.36	0.69	37.95	Not attributed	/
3	4.11	0.51	49.76	Not attributed	/
4	17.30	0.09	72.42	Astragali radix	Calycosin-7-glucoside
5	17.56	0.03	53.95	Glycyrrhizae radix et rhizoma	/
6	17.85	0.03	69.04	Glycyrrhizae radix et rhizoma	Liquiritin
7	23.34	0.23	34.79	Ginseng radix et rhizoma	Ginsenoside Re
8	23.50	0.23	53.44	Ginseng radix et rhizoma	Ginsenoside Rg ₁
9	29.30	0.17	53.51	Glycyrrhizae radix et rhizoma	/
10	29.67	0.07	43.56	Astragali radix	Calycosin
11	31.30	0.46	40.82	Glycyrrhizae radix et rhizoma	Glycyrrhizic acid
12	33.78	0.07	36.57	Ginseng radix et rhizoma	/
13*	34.25	0.05	61.78	Ginseng radix et rhizoma	Ginsenoside Rb ₁
14	34.65	0.05	38.97	Ginseng radix et rhizoma	/
15	35.06	0.05	47.87	Ginseng radix et rhizoma	/
16	36.02	0.05	71.96	Ginseng radix et rhizoma	/
17	37.31	0.05	92.89	Astragali radix	/
18	40.06	0.03	56.69	Ginseng radix et rhizoma	/
19	40.55	0.02	57.04	Glycyrrhizae radix et rhizoma	/

1 t_R , retention time.

2 RRT, relative retention time.

3 RPA, relative peak area.

* Reference peak.

the deviations in different batches of the decoction were caused not only by the non-common compounds but also by the contents of common compounds.

Method validation for the quantitation of multiple marker compounds in BYD by UPLC-DAD

Specificity. According to the Chinese pharmacopoeia [6–9] and the stability of the decoction, the following compounds were considered as marker compounds, separately representing the herbs composing the BYD: Ginsenosides Re, Rg₁ and Rb₁ for Ginseng radix et rhizoma; calycosin-7-glucoside and calycosin for Astragali radix; liquiritin, isoliquiritin apioside, isoliquiritin and glycyrrhizic acid for Glycyrrhizae radix et rhizoma; and cinnamic acid for Cinnamomi cortex.

Due to its higher sensitivity and precision compared to ELSD, DAD was applied in the quantitative method. As mentioned above, ginsenosides were detected under 203 nm; calycosin-7-glucoside, calycosin, liquiritin, glycyrrhizic acid and cinnamic acid responded well at 260 nm; and isoliquiritin apioside and isoliquiritin specifically responded at 360 nm.

As displayed in Fig. 3, good separation of the marker compounds in the complex decoction was obtained under this chromatographic condition, and each compound was specific to the corresponding herb. Ginsenosides Re and Rg₁ were difficult to separate due to their similar structures and polarities. Finally, they were quantitated together, as required by the Chinese pharmacopoeia [6].

Linearity, LOD, and LOQ. The regression equation, LOD and LOQ were estimated by the external standard method and are summarized in Table 5. Each marker compound had a wide linear range, and the correlation coefficient was greater than 0.9996. The LOD and LOQ suggested that the method was sufficiently sensitive for the quantitation of marker compounds in BYD.

Precision, Repeatability and Stability. The results of the apparatus precision, chromatographic condition repeatability, and sample stability are listed in Table 6. The RSDs of intra- and inter-day precision were under 1.0% and 2.0%, showing that the apparatus had a good performance in terms of consecutive analysis. The RSDs were no more than 2.1% in the validation of repeatability, showing that the chromatographic condition was repeatable. The RSDs were below 2.3% at 7 time points over 12 h, showing that the sample solution was stable within 12 h.

Accuracy. The accuracy results are shown in Table 7. The average recoveries of the marker compounds ranged from 81.25% to 102.86%, with the RSDs ranging from 0.3% to 1.1%.

The recoveries of isoliquiritin apioside, isoliquiritin and cinnamic acid were not perfect due to their low contents, resulting in greater deviation for these compounds than for other major compounds under the same sample preparation.

Because of the interference in the complex decoction, SPE was applied in the sample preparation of ginsenosides Re, Rg₁ and Rb₁ for good separation, and their recoveries were acceptable.

Fig. 3. UPLC-DAD chromatographic specificity of the quantitative method for BYD. The specificity of marker compounds of Ginseng radix et rhizome at 203 nm (A), Astragali radix at 260 nm (B), Glycyrrhizae radix et rhizome at 260 nm (C) and 360 nm (D), and Cinnamomi cortex at 260 nm (E). Mixed standard solution (a), decoctions of Ginseng (b), lack-Ginseng (c), BYD (d), Astragali (e), lack-Astragali (f), Glycyrrhizae (g), lack-Glycyrrhizae (h), Cinnamomi (i) and lack-Cinnamomi (j). Marker compounds of ginsenoside Re (1) and Rg₁ (2), ginsenoside Rb₁ (3), calycosin-7-glucoside (4), calycosin (5), liquiritin (6), glycyrrhizic acid (7), isoliquiritin apioside (8), isoliquiritin (9) and cinnamic acid (10)

Sample analysis for the quantitation of multiple marker compounds in BYD. The contents of marker compounds in 16 batches of BYD are listed in Table 8. Based on multiple batches of qualified crude herbs combined randomly, the content ranges of marker compounds in 16 batches of decoctions were wide—ginsenosides Re and Rg₁ ranged from 205.50 to 440.81 µg/mL, ginsenoside Rb₁ ranged from 38.66 to 276.12 µg/mL, calycosin-7-glucoside ranged from 52.20 to 185.73 µg/mL, calycosin ranged from 20.19 to 110.62 µg/mL, liquiritin ranged from 252.19 to 1028.32 µg/mL, isoliquiritin apioside ranged from 31.34 to 177.61 µg/mL, isoliquiritin ranged from 88.94 to 404.93 µg/mL, glycyrrhizic acid ranged from 441.25 to 1555.53 µg/mL, and cinnamic acid ranged from 2.39 to 42.10 µg/mL. Although the crude herbs sliced for the decoctions were qualified according to the Chinese pharmacopoeia, these results implied that it is a challenge to control the contents of marker compounds in BYD to within a stable range when using different sources of herbs.

DISCUSSION

The chromatographic fingerprint with multiple distinct peaks is helpful for evaluating the authenticity of the crude herbs composing the formula. Further, some of the distinct peaks that represent the marker compounds need to meet content requirements to ensure clinical efficacy. Ideally, the marker compounds should be the effective compounds. Network pharmacology could predict the possible connections between bioactive compounds and disease targets. However, the equivalence of disease definitions between modern medicine and the TCM, and the relationship of the precursor compounds in the formula with the effective compounds exposed *in vivo* still need further investigation and verification. Alternatively, the major compounds with bioactivity that were extracted in the dosage forms were considered as markers for quality control during the pharmaceutical process.

In the Chinese pharmacopoeia, cinnamaldehyde is the marker compound by which to control the quality of Cinnamomi cortex [9], which should be more than 1.5% (g/100 g crude herb). However, it is easily oxidized and volatilized. Our previous study showed that its content was from 2.3 to 6.1% in 15 batches of crude herbs but was not detectable after decoction. Therefore, cinnamic acid was considered as the marker compound representing Cinnamomi cortex in BYD due to its stability and quantifiable amount, though it is not the major compound of the crude herb. In consideration of the structural similarity and transformation between the two compounds, it was reasonable to add cinnamic acid as another marker compound by which to control the quality of Cinnamomi cortex. Similarly, astragaloside A, which represents Astragali radix [7], was replaced by calycosin-7-glucoside and calycosin because of the poor dissolution of astragaloside A in the decoction.

Table 5. Regression equation, correlation coefficient (r), linear range, LOD and LOQ of the marker compounds in BYD

Herbs	Marker compounds	Regression equation (mg/mL)	r	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Ginseng radix et rhizoma	Ginsenoside Re and Rg ₁	$y = 2979.4x + 11.636$	0.9996	25.01–400.16	6.67	20.01
	Ginsenoside Rb ₁	$y = 2210.0x + 19.038$	0.9999	25.01–400.16	0.67	2.22
Astragali radix	Calycosin-7-glucoside	$y = 31964x + 58.017$	0.9998	2.02–505.00	0.02	0.08
	Calycosin	$y = 40231x + 55.259$	0.9999	2.02–505.00	0.82	2.46
Glycyrrhizae radix et rhizoma	Liquiritin	$y = 8976.3x + 16.934$	0.9998	2.02–505.00	0.05	0.16
	Isoliquiritin apioside	$y = 26157x + 40.075$	0.9998	2.02–505.00	0.12	0.36
	Isoliquiritin	$y = 15806x + 24.269$	0.9998	0.80–200.50	0.21	0.63
	Glycyrrhizic acid	$y = 7281.3x + 10.521$	0.9999	2.00–500.00	0.05	0.14
Cinnamomi cortex	Cinnamic acid	$y = 55644x + 125.86$	0.9996	2.00–500.00	0.01	0.04

Table 6. Apparatus precision, chromatographic condition repeatability and sample stability of the quantitative method for BYD

Marker compounds	Precision (RSD, %)		Repeatability (RSD, %, n = 6)	Stability (RSD, %, 7 time points)
	Intra-day (n = 6 per day)	Inter-day (n = 18 in 3 d)		
Ginsenoside Re and Rg ₁	0.4	1.9	0.3	1.2
Ginsenoside Rb ₁	1.0	1.5	1.0	2.3
Calycosin-7-glucoside	0.3	2.0	0.5	2.3
Calycosin	0.2	0.4	0.6	0.6
Liquiritin	0.5	1.9	2.1	0.6
Isoliquiritin apioside	0.4	1.1	1.0	0.9
Isoliquiritin	0.4	1.1	1.4	0.8
Glycyrrhizic acid	0.4	1.5	0.6	0.8
Cinnamic acid	0.2	1.7	0.7	1.7

Table 7. Average recoveries of the quantitative method for BYD (n = 3)

Marker compounds	Original amounts (µg)	Spiked amounts (µg)	Detected amounts (µg)	Recovery (%)	RSD (%)
Ginsenoside Re and Rg ₁	650.0	520.0	1.15×10^3	96.35	1.1
		650.0	1.22×10^3	88.15	0.8
		810.0	1.41×10^3	94.20	0.6
Ginsenoside Rb ₁	145.0	116.0	252.0	92.24	0.5
		145.0	279.0	92.41	0.6
		174.0	308.0	93.68	0.6
Calycosin-7-glucoside	60.0	48.0	106.0	95.83	0.8
		60.0	117.0	95.00	0.9
		72.0	129.0	95.83	0.5
Calycosin	20.0	17.5	38.0	102.86	0.6
		20.0	39.0	95.00	0.6
		25.0	45.0	100.00	0.4
Liquiritin	95.0	80.0	170.0	93.75	0.6
		95.0	178.0	87.37	0.7
		110.0	199.0	94.55	0.8
Isoliquiritin apioside	15.0	14.0	28.0	92.86	0.5
		15.0	28.0	86.67	0.3
		18.0	31.0	88.89	0.8
Isoliquiritin	16.0	15.0	29.0	86.67	0.5
		16.0	29.0	81.25	0.6
		18.0	31.0	83.33	0.5
Glycyrrhizic acid	180.0	168.0	337.0	93.45	0.5
		180.0	338.0	87.78	0.6
		216.0	378.0	91.67	0.5
Cinnamic acid	6.6	5.2	11.0	84.62	0.7
		6.6	12.0	81.82	0.7
		7.6	13.0	84.21	0.9

Table 8. Contents of the marker compounds, paste rates and similarities of 16 batches of BYD

Batch No.	Contents (µg/mL)									Paste rates ² (%)	Similarities ³	
	Ginsenoside Re and Rg ₁	Ginsenoside Rb ₁	Calycosin- 7-glucoside	Calycosin	Liquiritin	Isoliquiritin apioside	Isoliquiritin	Glycyrrhizic acid ¹	Cinnamic acid		DAD	ELSD
S1	364.36	212.23	101.21	110.62	430.17	62.88	163.47	686.19	11.78	20.90	0.943	0.862
S2	370.40	54.50	115.16	65.13	1002.41	99.12	380.09	1070.55	10.87	21.36	0.973	0.907
S3	440.81	276.12	104.16	87.30	818.33	158.07	183.58	1263.34	15.63	21.44	0.989	0.963
S4	350.96	152.78	109.72	50.42	494.41	154.05	219.59	1555.53	18.55	20.08	0.970	0.959
S5	253.73	134.95	113.38	20.19	299.47	98.44	102.99	942.05	13.63	20.01	0.956	0.945
S6	300.62	110.42	185.73	84.92	340.93	177.61	140.47	1486.45	42.10	20.00	0.951	0.906
S7	296.02	45.81	59.47	58.62	613.79	84.76	271.05	956.80	25.43	21.24	0.972	0.970
S8	289.88	107.35	91.53	50.88	386.32	154.26	147.28	1232.26	34.90	20.9	0.960	0.950
S9	231.98	43.00	121.89	63.05	325.09	114.18	112.77	765.06	15.59	20.11	0.951	0.955
S10	433.40	133.95	79.78	96.58	1028.32	92.01	404.93	1129.51	7.43	21.14	0.957	0.941
S11	293.54	137.39	61.76	48.53	347.06	113.78	125.05	1083.44	10.89	20.94	0.971	0.942
S12	298.77	38.66	63.74	43.27	787.31	77.38	280.23	858.12	2.39	19.15	0.945	0.893
S13	241.14	74.13	54.80	48.04	356.80	31.34	138.76	441.25	11.51	20.51	0.888	0.916
S14	414.33	119.38	161.42	72.49	811.08	64.73	344.48	677.31	25.96	22.22	0.935	0.889
S15	205.50	42.37	52.20	37.11	252.19	70.98	88.94	699.13	15.05	20.18	0.976	0.960
S16 ⁴	320.12	122.46	101.86	66.11	577.80	104.31	227.26	1004.65	17.14	21.85	0.997	0.999
Mean ± SD	319.03 ±	112.20	98.40 ±	62.48 ±	552.91 ±	103.57 ±	206.91 ±	989.80 ±	17.45 ±	20.68 ±		
from S1	73.82	±67.87	39.02	24.21	267.58	41.79	104.57	314.14	10.55	0.77		
to S15												
RSD (%)	23.2	60.5	39.7	38.8	48.4	40.4	50.5	31.7	60.45	3.7		

¹The calibration curve of glycyrrhizic acid was established in the form of ammonium salt, but the content was converted to glycyrrhizic acid.

²Paste rate was calculated as the amount percentage of dried paste from the decoction to the total formula.

³The similarities were calculated by comparing the fingerprints of 16 batches of BYD to the characteristic fingerprints detected by DAD and ELSD, respectively.

⁴S1 to S15 were made from the four kinds of herbs selected randomly from each of the 15 batches. S16 was composed by mixing every batch of each individual herb in equal amount.

Fig. 4. Research and development procedure of transformation from traditional to modern dosage forms, take *Bao-Yuan Decoction* as an example. Pictures used in the figure were obtained from photo gallery of Baidu. Formulas in the traditional Chinese medicine (TCM) have been used in the clinic for a long time due to their safety and effectiveness. To guarantee the clinical efficacy of modern dosage forms, it is a key point to ensure the consistency of quality attributes in modern forms with those in traditional formulas. However, the complexity of the herbs composing the formula increases the difficulty to clarify the effective compounds that should be considered perfect markers for quality control. Moreover, the effective compounds are not only the small molecules but also the macro molecules, such as polysaccharides. Therefore, it is reasonable to evaluate the quality attributes in multiple-dimensions. Due to formulas in the TCM are originally prepared from crude herbs that exist compound variation in quantity and amounts from different producing areas, ascertaining the quality value transfer in the chain from the crude herbs to the final dosage forms would support the quality control of the modern dosage forms

As illustrated in Fig. 4, the traditional dosage forms of the formula that have been used for a long time due to their safety and effectiveness were taken as a reference, and its quality attributes provided a good guideline by which to develop and control the modern dosage forms. This study was focused on the evaluation of the small molecules in BYD, which were separated and detected by the commonly equipped LC-DAD/ELSD instrument. Moreover, the Ginseng radix et rhizoma, Astragali radix and Glycyrrhizae radix et rhizoma that composed the formula were rich in polysaccharides. It has already been demonstrated that polysaccharides are involved in immune regulation, which coincides with treatments using BYD, to tonify the deficiency of Qi, as interpreted by the TCM [10–12]. In consequence, our team is now conducting the quantification of polysaccharides of BYD, which will be a good supplement in the systematic quality control of the decoction.

Additionally, the formula in the TCM was originally prepared from crude herbs. Ascertaining the quality value transfer in the chain from the crude herbs to the final dosage forms would support the quality control of the modern dosage forms.

CONCLUSION

In the present study, the commonly equipped UPLC-DAD/ELSD instrument was initially applied to develop the analytical method for the qualitative fingerprints and simultaneous quantitation of multiple marker compounds of

BYD prepared according to the traditional procedure. Based on 16 batches of BYD prepared from qualified crude herbs from major or genuine producing areas, the characteristic fingerprints and the content ranges of marker compounds were obtained, which provide vital connections in the quality value transfer from the crude herbs to the modern dosage forms and will be a key standard upon which to develop, evaluate and control the quality of the modern dosage forms. This strategy will be a good reference for developing the formula in the TCM into modern dosage forms in an operational and applicable way.

Conflict of Interests: All the listed authors have read and approved this article. There is no financial conflict of interest with the authors of this article. This manuscript has not been submitted for possible publication to another journal.

SUPPLEMENTARY MATERIAL

The following is the supplementary data to this article:

The online version of this article offers supplementary material <https://doi.org/10.1556/1326.2020.00858>.

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