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Development of a Simultaneous Analysis Method for Quality Control of a Traditional Herbal Formula, Daeshiho-Tang, Using 10 Marker Components

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Abstract: Daeshiho-tang (DSHT) is a traditional herbal formula consisting of six herbal medicines: *Bupleurum falcatum* L., *Scutellaria baicalensis* Georgi, *Paonia lactiflora* Pall., *Pheum palmatum* L., *Poncirus trifoliata* (L.) Raf., and *Pinellia ternate* (Thunb.) Makino. In this study, we developed a simultaneous analysis method based on high-performance liquid chromatography for the quality control of DSHT. Chromatographic separation of 10 marker components (gallic acid, albiflorin, paeoniflorin, naringin, benzoic acid, baicalin, poncirin, wogonoside, baicalein, and wogonin) was achieved using a water–acetonitrile system as the mobile phase with a SunFire C18 reversed-phase column. The developed analytical method was validated with respect to linearity, limit of detection, limit of quantitation, recovery, and precision. Among the 10 markers of DSHT in the established assay, baicalin, the main compound of *Scutellaria baicalensis* Georgi, was present in the highest concentration (36.86–46.17 mg/g). The validated assay will be useful for the quality control of DSHT.

Keywords: simultaneous analysis; quality control; traditional herbal formula; daeshiho-tang

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1. Introduction

Traditional Korean medicine (TKM), traditional Chinese medicine (TCM), and Kampo medicine (KM) are attracting increasing interest as because their multicomponent and multitarget characteristics that enable the therapeutic or adjuvant treatment of a range of diseases [1]. In addition, given that the active ingredients are usually obtained by extracting natural herbal medicines with water without artificial additives, there are few side effects. Thus, TCM, TKM, or KM are seeing widespread use and increasing interest in complementary and alternative medicines [2].

Daeshiho-tang (DSHT, Dachaihu-tang in Chinese, or Daisaiko-to in Japanese) is a well-known traditional herbal formula that was first recorded in Shang-han-lun (傷寒論), a textbook by Zhang Zhongjing, and also included in Donguibogam (東醫寶鑑) and Bangyakhappyeon (方藥合編) in Korea [3,4]. According to the Bangyakhappyeon, DSHT is composed of six medicinal herbs: *Bupleurum falcatum* L., *Scutellaria baicalensis* Georgi, *Paonia lactiflora* Pall., *Pheum palmatum* L., *Poncirus trifoliata* (L.) Raf., and *Pinellia ternate* (Thunb.) Makino, and typically prescribed for the treatment of headache, chills, fever, hard stool, ruddy urine, and nervousness [4–6].

Studies on the biological efficacy of DSHT have been reported for analgesic, antipyretic, hepatoprotective, hypertension, obesity, hypercholesterolemia, and atherosclerosis action [3,7,8]. Li et al. reported chromatographic separation conditions that could be used efficiently to separate four isomers in human urine administered with DSHT by using high-performance liquid chromatography (HPLC) [9,10]. Iizuka et al. presented HPLC profiling for DSHT [8], and Li et al. performed a simultaneous analysis study on eight major components (paeoniflorin, naringin, sennoside A, baicalin, baicalein, saikosaponin A, rhein, and emodin) in DSHT [11]. However, these assays are limited by the long analysis

time (90 min) required to separate the eight components and were primarily focused on establishing the conditions for the separation of isomers and profiling.

The constituent herbal medicines of DSHT contain many phytochemical components, namely: triterpenoids (saikosaponin A) from *B. falcatum* [12], flavonoids (baicalin, baicalein, and wogonin) from *S. baicalensis* [13], phenolic compounds (gallic acid and benzoic acid) and monoterpenoids (albiflorin and paeoniflorin) from *P. lactiflora* [14], anthraquinones (physcion and chrysophanol) from *P. palmatum* [15], flavonoids (naringin and poncirin) from *P. trifoliata* [16], and phenolic compounds (homogentisic acid and 3,4-dihydroxybenzaldehyde) from *P. ternate* [17].

In this study, a chromatographic analysis method based on HPLC separation coupled with diode array detection (DAD) was developed to target the 10 marker compounds (gallic acid, albiflorin, paeoniflorin, and benzoic acid from *P. lactiflora*, naringin and poncirin from *P. trifoliata*; and baicalin, wogonoside, baicalein, and wogonin from *S. baicalensis*) within a relatively short time for the consistent quality control of DSHT.

2. Materials and Methods

2.1. Plant Materials

Six raw herbs (*B. falcatum*, *S. baicalensis*, *P. lactiflora*, *P. palmatum*, *P. trifoliata*, and *P. ternate*) constituting DSHT were purchased from a commercial herbal medicine market, Kwangmyungdag Medicinal Herbs (Ulsan, Korea), in November 2017. The identities of the herbs (2017–KE61–1 to 2017–KE61–6) were verified by morphological examination by Dr. Goya Choi, Korea Institute of Oriental Medicine (KIOM, Naju, Korea), according to the guidelines of "The Dispensatory on the Visual and Organoleptic Examination of Herbal Medicine" [18], and samples have been deposited with KIOM. The scientific species name of each medicinal herb was verified on "The Plant List" (www.theplantlist.org, accessed on 28 September 2021) website.

2.2. Preparation of DSHT Water Decoction

DSHT water decoction was made by KIOM according to the composition detailed in the Bangyakhappyeon [4]. Namely, as shown in Table S1, after mixing the six herbs by weight ratio, 50 L of water was added and the mixture was extracted at 100 °C for 2 h with an electric extractor (COSMOS-660, Kyungseo E&P, Incheon, Korea). The extract solution was then filtered through a sieve (53 μm) and freeze-dried (PVTFD100R, IlShinBioBase, Dongducheon, Korea). The lyophilized extract (yield 25.6%, 1280.8 g) was stored at 4 °C until analysis.

2.3. Chemicals and Reagents

Then, reference standard compounds (Figure S1) were selected as marker components for the quality control of DSHT using HPLC. The compounds were purchased from commercial companies: gallic acid (CAS No., 149-91-7, Cat no. G7384, purity 100.0%), naringin (CAS no. 10236-47-2, Cat no. 71162, purity 95.0%), benzoic acid (CAS no. 65-85-0, Cat no. 242381, purity 99.9%), and baicalein (CAS no. 491-67-8, Cat no. 2465119, purity 98.0%) from Merck KGaA (Darmstadt, Germany); albiflorin (CAS no. 39011-90-0, Cat no. 016-22201, purity 99.8%), baicalin (CAS no. 21967-41-9, Cat no. 024-15691, purity 98.0%), and wogonin (CAS no. 632-85-9, Cat no. 236-02321, purity 98.9%) from Fujifilm Wako Pure Chemical Co. (Osaka, Japan); and paeoniflorin (CAS no. 23180-57-6, Cat no. DR10579, purity 99.4%), poncirin (CAS no. 14941-08-3, Cat no. DR11185, purity 98.9%), and wogonoside (CAS no. 51059-44-0, Cat no. DR10630, purity 98.9%) from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China).

HPLC-grade solvents, methanol, acetonitrile, and water, and the reagent, formic acid, were purchased from J.T.Baker (Phillipsburg, NJ, USA) and Fujifilm Wako Pure Chemical Co. (Osaka, Japan).

2.4. HPLC Conditions for Simultaneous Analysis of the 10 Marker Components in DSHT

HPLC analysis conditions for the quantification of the 10 components (gallic acid, albiflorin, paeoniflorin, naringin, benzoic acid, baicalin, poncirin, wogonoside, baicalein, and wogonin) in DSHT were applied as described in the reported protocols [19]. Briefly, a Prominence LC-20A series HPLC system (Shimadzu, Kyoto, Japan) consisting of two pumps, autosampler, degasser, column oven, and DAD was used. The system was controlled by LCSolution software (Ver. 1.24, SP1, Shimadzu, Kyoto, Japan). Details of the HPLC analysis conditions are summarized in Table S2.

2.5. Preparation of Sample and Standard Solutions for HPLC Analysis of the 10 Marker Components in DSHT

To quantify the 10 components in DSHT, a lyophilized DSHT sample (200 mg) was measured accurately, methanol (70%; 20 mL) was added, and the sample was submitted to ultrasonic extraction at room temperature for 60 min. The sample solution prepared for quantification of baicalin was diluted 10-fold and analyzed. A stock solution of each reference standard compound was prepared at a concentration of 1000 μg/mL in methanol. The prepared sample solution and standard solutions were filtered through a 0.2-μm membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) before HPLC analysis.

2.6. Method Validation of the HPLC Assay for Quality Control of DSHT

To validate the developed HPLC method, the linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision were evaluated. Using the standard solution, LOD and LOQ were calculated from Equations (1) and (2).

$$\mathrm{LOD} = 3.3 \times \sigma / S, \quad (1)$$

$$\mathrm{LOD} = 10 \times \sigma / S, \quad (2)$$

where σ and S are the standard deviation of the y-intercept and the slope of the calibration curve, respectively.

The extraction recovery was tested with the standard addition method; that is, standard solutions for three different concentrations (low, medium, and high) of known markers were added to the DSHT sample solution (200 mg), and the samples were pretreated according to the preparation of sample solution described in Section 2.5. The extraction recovery was calculated from Equation (3).

$$\text{Extraction recovery } (\%) = \text{measured concentration/spiked concentration } \times 100\% \quad (3)$$

Precision tests, intraday precision, interday precision, and repeatability were evaluated as relative standard deviation (RSD) values and calculated from Equation (4).

$$\mathrm{RSD}\ (\%) = \text{standard deviation/mean } \times 100\% \quad (4)$$

Intraday and interday precisions were evaluated by repeatedly measuring a standard solution containing the 10 markers five times on a single day and on three consecutive days, respectively; repeatability was evaluated by measuring retention time and peak area of a standard solution containing the 10 markers six times.

3. Results and Discussion

3.1. Selection of Marker Components and Optimization of HPLC–DAD Conditions for Quality Control of DSHT

To determine the optimal marker components for the quality assessment of DSHT, HPLC–DAD analysis was conducted on each component herbal medicine in DSHT to establish the major ingredients. To this end, 16 compounds were investigated: saikosaponin A from *B. falcatum*; baicalin, wogonoside, baicalein, and wogonin from *S. baicalensis*; gallic acid, albiflorin, benzoic acid, benzoylpaeoniflorin, gallic acid, and paeoniflorin from

P. lactiflora; chrysophanol and physcion from *P. palmatum*; naringin and poncirin from *P. trifoliata*; and 3,4-dihydrobenzaldehyde and homogentisic acid from *P. ternate*. As shown in Figure S2, 10 components (gallic acid, albiflorin, paeoniflorin, naringin, benzoic acid, baicalin, poncirin, wogonoside, baicalein, and wogonin) in the DSHT sample were detected out of 16 components investigated, and these 10 compounds were selected as marker components for quality control of DSHT through HPLC analysis.

After comparing the column temperatures (30, 35, 40, and 45 °C) and the reversed-phase columns (4.6 mm × 250 mm, 5 μm) such as SunFire (Waters, Milford, MA, USA), Gemini (Phenomenex, Torrance, CA, USA), Capcell Pak UG80 (Shiseido, Tokyo, Japan), and Optima Pak (RS Tech. Co., Daejeon, Korea) using water–acetonitrile (both containing formic acid) as the mobile phase, optimal conditions for analysis were found with a SunFire column maintained at 40 °C. Using the optimized HPLC–DAD assay, the 10 marker components (gallic acid, albiflorin, paeoniflorin, naringin, benzoic acid, baicalin, poncirin, wogonoside, baicalein, and wogonin) in the DSHT sample were eluted with the resolution ≥2.13 and the retention factor ≥1.25 at 6.49, 16.45, 17.32, 21.11, 23.22, 26.32, 26.74, 29.98, 32.34, and 38.07 min, respectively (Figure 1).

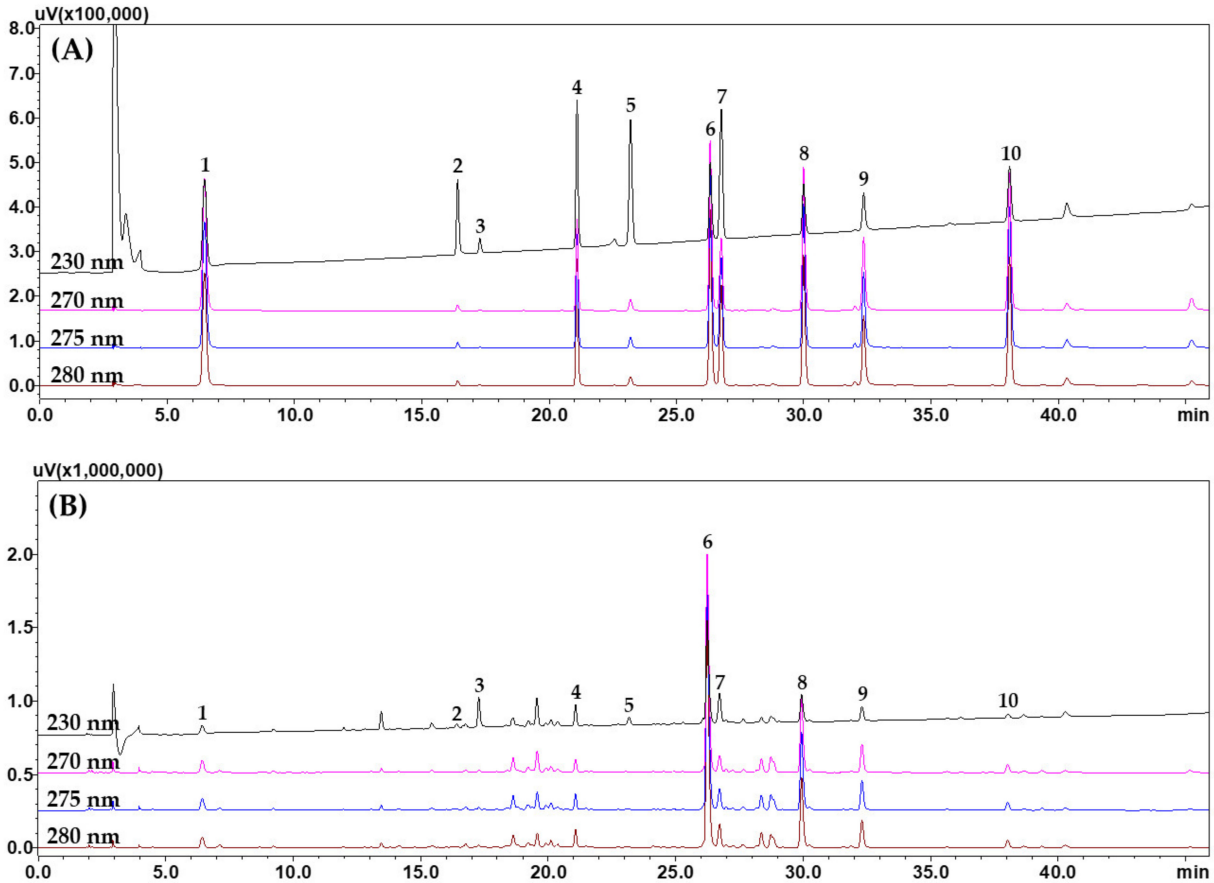

Figure 1. *Cont.*

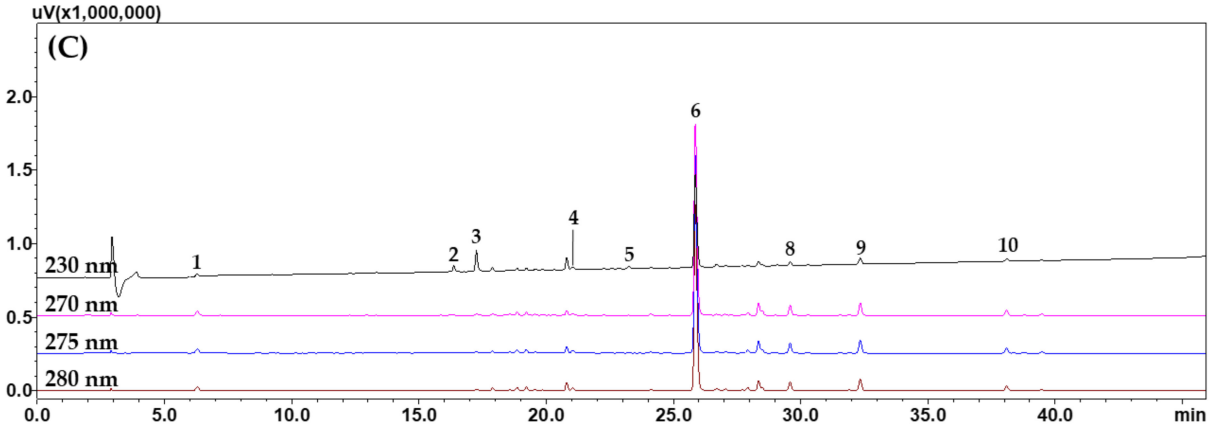

Figure 1. Representative HPLC chromatograms of (**A**) standard solution with 10 marker compounds, (**B**) DSHT sample manufactured by KIOM, and (**C**) commercial DSHT sample distributed and manufactured by a Korean pharmaceutical company. The peaks are gallic acid (**1**), albiflorin (**2**), paeoniflorin (**3**), naringin (**4**), benzoic acid (**5**), baicalin (**6**), poncirin (**7**), wogonoside (**8**), baicalein (**9**), and wogonin (**10**).

3.2. Method Validation of the HPLC Assay for Quality Control of DSHT

The regression equation ($y = ax + b$) for the calibration curve of each marker measured at seven concentration levels was calculated using the area (y)-to-concentration (x, μg/mL) ratio with standard solutions. As shown in Table 1, all coefficient of determination (r^2) values representing linearity in the prepared calibration curve were ≥0.9990, indicating good linearity, and LOD and LOQ were calculated to be 0.07–0.68 μg/mL and 0.21–2.07 μg/mL using Equations (1) and (2) in Section 2.6, respectively. Recovery studies of all markers calculated from Equation (3) were determined to be 94.68–105.86%, within 3.0% RSD (Table 2). The RSD value of precision was calculated from Equation (4). The repeatability of the method was established by measuring the retention time and peak area in six repeated assays. The results confirmed good repeatability of the assay system, with RSD values for retention time and peak area of 0.02–0.73% and 0.50–0.98%, respectively (Tables S3 and S4). The RSD values of intraday and interday precisions were measured to be <1.50% (with an accuracy of 96.07–105.85%) and <3.0% (with an accuracy of 96.50–106.19%), respectively, as shown in Table 3. These validation results confirm that the HPLC–DAD assay developed for quality control of DSHT using 10 marker components is appropriate.

Table 1. Linear range, regression equation, r^2, LOD, and LOQ of the 10 markers for simultaneous analysis by HPLC (n = 3).

Analyte	Detection Wavelength (nm)	Linear Range (μg/mL)	Regression Equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	270	1.56–100.00	$y = 32{,}987.60x - 4335.65$	1.0000	0.09	0.27
Albiflorin	230	1.56–100.00	$y = 11{,}300.32x - 8422.51$	0.9999	0.20	0.62
Paeoniflorin	230	3.13–200.00	$y = 19{,}843.02x - 39{,}497.69$	0.9998	0.13	0.39
Naringin	280	3.13–200.00	$y = 17{,}455.31x - 24{,}581.58$	0.9999	0.20	0.60
Benzoic acid	230	0.78–50.00	$y = 49{,}895.95x - 4450.86$	0.9999	0.04	0.12
Baicalin	275	3.13–200.00	$y = 30{,}316.63x - 33{,}142.13$	0.9999	0.25	0.76
Poncirin	280	4.69–300.00	$y = 18{,}470.06x - 39{,}998.20$	0.9999	0.32	0.97
Wogonoside	275	4.69–300.00	$y = 53{,}437.97x - 65{,}613.43$	0.9999	0.35	1.07
Baicalein	275	3.13–200.00	$y = 51{,}497.99x - 205{,}774.62$	0.9990	0.68	2.07
Wogonin	275	0.47–30.00	$y = 64{,}440.29x - 1976.11$	0.9999	0.07	0.21

Table 2. Extraction recovery (%) of the 10 markers in the developed HPLC assay (n = 5).

Analyte	Spiked Conc. (μg/mL)	Measured Conc. (μg/mL)	Recovery (%)	SD	RSD (%)
Gallic acid	6.00	31.72	98.90	0.58	0.59
	15.00	40.84	100.34	0.84	0.84
	30.00	56.61	102.73	0.60	0.59
Albiflorin	4.00	19.29	98.11	0.76	0.78
	10.00	25.50	101.36	2.20	2.17
	20.00	34.93	97.83	1.57	1.61
Paeoniflorin	16.00	91.01	98.16	2.64	2.69
	40.00	115.71	101.01	0.82	0.81
	80.00	151.04	94.68	0.96	1.01
Naringin	10.00	60.20	99.50	0.76	0.76
	25.00	75.05	99.19	1.16	1.17
	50.00	102.52	104.53	0.73	0.70
Benzoic acid	2.00	10.93	97.93	0.80	0.82
	5.00	13.96	99.94	2.24	2.24
	10.00	18.95	99.82	2.00	2.00
Baicalin	10.00	56.43	102.95	1.28	1.24
	25.00	71.81	102.70	0.92	0.90
	50.00	97.33	102.38	0.28	0.28
Poncirin	16.00	91.09	100.23	1.64	1.64
	40.00	117.40	105.86	1.04	0.99
	80.00	155.60	100.68	0.33	0.33
Wogonoside	16.00	99.93	99.37	1.05	1.06
	40.00	124.48	101.12	0.30	0.30
	80.00	165.32	101.62	0.21	0.21
Baicalein	8.00	47.77	99.97	1.37	1.37
	20.00	59.84	100.37	0.58	0.58
	40.00	79.70	99.83	0.96	0.96
Wogonin	2.00	9.78	95.90	0.30	0.32
	5.00	12.90	100.74	1.89	1.88
	10.00	18.39	105.33	2.27	2.15

Table 3. Precision date of the 10 markers in the developed HPLC assay (n = 5).

Analyte	Conc. (μg/mL)	Intraday			Interday		
		Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%) [a]	Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)
Gallic acid	12.50	12.01	1.07	96.07	12.24	2.98	97.96
	25.00	24.13	0.30	96.50	24.12	2.06	96.50
	50.00	48.30	0.28	96.60	48.26	1.02	96.53
Albiflorin	12.50	12.25	1.66	98.03	12.43	1.47	99.46
	25.00	25.19	0.28	100.78	25.03	1.25	100.13
	50.00	50.75	1.89	101.50	50.55	1.25	101.11
Paeoniflorin	25.00	25.34	1.10	101.35	25.34	0.90	101.35
	50.00	52.47	1.09	104.95	51.27	2.01	102.55
	100.00	102.42	0.90	102.42	102.71	0.73	102.71
Naringin	12.50	13.07	1.42	104.53	13.19	1.47	105.48
	25.00	25.32	0.78	101.29	25.37	1.15	101.47
	50.00	50.10	0.32	100.20	50.10	0.76	100.20
Benzoic acid	12.50	12.80	0.35	102.40	12.94	1.30	103.55
	25.00	26.27	0.71	105.10	26.34	0.97	105.34
	50.00	52.93	0.71	105.85	52.76	1.33	105.52

Table 3. *Cont.*

Analyte	Conc. (μg/mL)	Intraday			Interday		
		Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%) [a]	Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)
Baicalin	12.50	12.47	1.03	99.74	12.62	1.54	100.94
	25.00	24.46	0.25	97.83	24.65	1.26	98.59
	50.00	48.64	0.33	97.28	48.78	0.99	97.55
Poncirin	25.00	26.43	0.70	105.72	26.55	0.92	106.19
	50.00	49.80	0.45	99.59	49.68	0.58	99.35
	100.00	96.86	0.33	96.86	97.11	0.64	97.11
Wogonoside	25.00	26.44	0.76	105.74	26.68	1.32	106.72
	50.00	52.89	0.46	105.78	52.92	1.04	105.85
	100.00	105.29	0.43	105.29	104.84	0.85	104.84
Baicalein	12.50	13.16	0.65	105.28	12.99	1.13	103.94
	25.00	25.26	0.35	101.05	25.26	1.12	101.04
	50.00	50.57	0.65	101.14	50.54	1.17	101.08
Wogonin	7.50	7.57	0.41	100.87	7.60	0.78	101.29
	15.00	15.34	0.50	102.25	15.40	0.65	97.92
	30.00	30.22	0.63	100.74	30.21	0.39	100.69

[a] Accuracy = measured concentration/concentration × 100.

3.3. Simultaneous Quantification of the 10 Marker Analytes in DSHT Samples Using HPLC

The HPLC assay developed for the quality control of DSHT in this study was applied for the simultaneous quantitative analysis of DSHT samples. As shown in Table 4, the 10 selected marker compounds in the DSHT sample prepared by KIOM were detected at concentrations of 0.77–46.17 mg/g. Furthermore, the DSHT granules produced and distributed by a Korean pharmaceutical company were also analyzed using the established analytical method. In this case, the marker compounds were detected at levels up to 39.34 mg/g; however, poncirin, a major compound of *P. trifoliata*, was not detected in this sample. It is considered that this is due to the origin of the P. trifoliata, which is a component of DSHT [20]. Of the marker compounds assessed, baicalin, which is known to be the main compounds of *S. baicalensis*, was detected at 36.86–46.17 mg/g and was confirmed as the major component in the DSHT samples.

Table 4. Concentrations of the 10 markers in the DSHT samples determined with the HPLC–DAD assay (n = 3).

Analyte	Amount (mg/g Freeze-Dried Sample)								Origin [c]
	Sample 1 [a]				Sample 2 [b]				
	Batch 1		Batch 2		Batch 1		Batch 2		
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	
Gallic acid	2.62	0.46	2.62	0.52	0.84	0.74	0.84	0.25	PL
Albiflorin	1.52	1.02	1.48	1.50	2.41	0.05	2.39	0.24	PL
Paeoniflorin	7.72	0.31	7.75	0.43	4.94	0.04	4.93	0.16	PL
Naringin	5.05	0.35	5.03	0.35	1.10	0.26	1.09	0.54	PT
Benzoic acid	0.96	0.37	0.96	1.17	0.29	0.29	0.29	0.17	PL
Baicalin	46.17	0.44	45.79	0.33	39.34	0.52	36.86	0.02	SB
Poncirin	7.57	0.07	7.53	0.28	ND [d]	–	ND	–	PT
Wogonoside	8.32	0.21	8.35	0.26	1.09	0.03	1.10	0.16	SB
Baicalein	3.77	0.47	3.78	0.49	1.78	0.16	1.77	0.05	SB
Wogonin	0.77	0.16	0.77	0.17	0.51	0.10	0.51	0.14	SB

[a] DSHT sample manufactured by KIOM. [b] DSHT sample of commercial products manufactured by a Korean pharmaceutical company. [c] PL—*P. lactiflora*; PT—*P. trifoliata*; SB—*S. baicalensis*. [d] Not detected.

4. Conclusions

In this study, an HPLC analysis method using major marker components was developed for quality control of DSHT, an herbal medicine prescription used in Soyangyangmyunghab-byung. The developed method was validated with respect to linearity, LOD, LOQ, recovery, and precision, and has been successfully applied to the simultaneous analysis of DSHT.

The method developed in this study will provide useful basic data for establishing analysis methods for the quality control of other TCMs, TKMs, and KMs in addition to DSHT.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app112110242/s1, Figure S1: Chemical structures of the 10 marker components in DSHT; Figure S2: HPLC chromatogram of the mixed 16 standard solution (A) and the DSHT sample (B). Gallic acid (**1**), homogentisic acid (**2**), 3,4-dihydroxybenzaldehyde (**3**), albiflorin (**4**), paeoniflorin (**5**), naringin (**6**), benzoic acid (**7**), baicalin (**8**), poncirin (**9**), benzoylpaeoniflorin (**10**), wogonoside (**11**), baicalein (**12**), saikosaponin A (**13**), wogonin (**14**), chrysophanol (**15**), and physcion (**16**); Table S1: Information and composition of DSHT; Table S2: HPLC analysis conditions for simultaneous determination of the 10 marker components in DSHT; Table S3: Repeatability of the retention times of the 10 markers in HPLC analysis (n = 6); Table S4: Repeatability for peak area of the 10 markers in HPLC analysis (n = 6).

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Conflicts of Interest: The authors declare no conflict of interest.

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