Article

Simultaneous Analysis to Evaluate the Quality of Insamyangpye–Tang Using High-Performance Liquid Chromatography–Photo Diode Array Detection

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Abstract: Insamyangpye–tang (ISYPT) is a traditional medicinal formula comprised of 13 herbs and has been used in East Asia to treat lung-related diseases. However, to our knowledge, no method of analysis for its quality control has been reported. In this study, a method of analysis for quality control of ISYPT was developed using high-performance liquid chromatography. Chromatographic separation, analysis, and assay verification were performed with a distilled water–acetonitrile mobile phase system, both containing 0.1% (*v/v*) trifluoroacetic acid, and a Gemini C18 analytical column (4.6 mm × 250 mm, 5 μm) using authentic standards for eight marker compounds. These marker constituents were detected simultaneously at 0.09–5.95 mg/g. The analysis method developed can be used for basic quality control of ISYPT.

Keywords: simultaneous analysis; quality control; insamyangpye–tang; HPLC–PDA

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1. Introduction

There is a growing interest and demand for traditional Korean medicine (TKM), traditional Chinese medicine (TCM), and Kampo medicine (KM) worldwide, especially in East Asian countries. In general, they are composed of two or more different herbal medicines and have both multi-component and multi-target features. Because they are known to have few side effects, traditional medicines have the advantage in that they can be used widely to treat and prevent various diseases [1–3]. To manage the quality of TKM, TCM, and KM for their continued use, standardization studies are required.

Insamyangpye–tang (ISYPT) is a traditional Korean medicinal formula comprised of 13 different types of herbal medicines (*Bupleurum falcatum* L., *Morus alba* L., *Poria cocos* Wolf, *Schisandra chinensis* Baill., *Fritillaria thunbergii* Miq., *Prunus armeniaca* L., *Poncirus trifoliata* Rafin., *Platycodon grandiflorum* A.DC., *Panax ginseng* C.A.Mey., *Equus asinus* L., *Glycyrrhiza uralensis* Fisch., *Zingiber officinale* Rosc., and *Ziziphus jujuba* Mill.). ISYPT is an herbal prescription representative of those used to treat lung-related diseases, such as pulmonary tuberculosis and bronchitis [4].

The main constituents of herbal medicines comprising ISYPT are triterpene saponins, saikosaponin A, C, and D from *B. falcatum* [5]; stilbenoids, mulberroside A, and flavones, kuwanon G and morusin from *M. alba* [6]; lanostane-type triterpenoids, pachymic acid from *P. cocos* [7]; lignans, schizandrol A and gomisin A from *S. chinensis* [8]; alkaloids, peimine and peiminine from *F. thunbergii* [9]; cyanogenic glycoside compounds, amygdalin from *P. armeniaca* [10]; flavonoid glucosides, naringin, didymine, and poncirin from *P. trifoliata* [11]; triterpene saponins, platycodin D and platycodin D2 from *P. grandiflorum* [12]; triterpene saponins, ginsenoside Rb1, and ginsenoside Rg1 from *P. ginseng* [13]; amino acids, glycine and L-proline from *Equus asinus* [9]; triterpene saponins, glycyrrhizic acid, and flavanone glucosides, liquiritoside and liquiritin apioside from *G. uralensis* [14]; phenolic compounds, 6-gingerol from *Z. officinale* [15]; and C-glycoside flavonoids, spinosin from *Z. jujuba* [16].

In the present study, for the first time to our knowledge, a method of analysis for quality control of ISYPT to assure consistency was developed using a high-performance liquid chromatography–photodiode array detection (HPLC–PDA) system for simultaneous measurement of eight marker compounds. The eight marker compounds selected were mulberroside A (MBRSA) as found in *M. alba*, liquiritin apioside (LQRTA), liquiritoside (LQRTS), and glycyrrhizic acid (GCRZA) as found in *G. uralensis*, naringin (NRG), didymnin (DDM), and poncirin (PCR) as found in *P. trifoliata*, and schizandrol A (SZDRA) as found in *S. chinensis*.

2. Materials and Methods

2.1. Plant Materials

The 13 types of medicinal herbs that make up ISYPT, as shown in Table S1, were obtained from a specialized medicinal herb store, Kwangmyungdag Medicinal Herbs (Ulsan, Korea), in 2018. All of them were evaluated morphologically by Dr. Goya Choi, Herbal Medicine Resources Research Center (HMRRC), Korea Institute of Oriental Medicine (KIOM, Naju, Korea), according to National Institute of Food and Drug Safety Evaluation guidelines, the Korean Pharmacopoeia, and the Korean Herbal Pharmacopoeia [17–19]. Samples of each (2018–CA03–1 to 2018–CA03–13) have been deposited in the KIOM herbarium.

2.2. Chemicals and Reagents

Authentic standards of the eight marker compounds (Figure S1) used to assess the quality of ISYPT were supplied by reference standard manufacturers: namely, MBRSA (98.1%) from EnsolBioSciences (Daejeon, Korea); NRG (95.0%) from Merck (Darmstadt, Germany); LQRTA ($\geq 98.0\%$) and PCR (98.9%) from Shanghai Sunny Biotechnology (Shanghai, China); LQRTS (99.6%), GCRZA (99.1%), and SZDRA (99.3%) from Chengdu Biopurify (Chengdu, China); DDM (98.2%) from ChemFaces (Wuhan, China).

Methanol (MeOH), acetonitrile (ACN), distilled water (DW), and trifluoroacetic acid (TFA, $\geq 99.0\%$) were all HPLC analysis-grade and provided from JT Baker (Phillipsburg, NJ, USA) and Merck (Darmstadt, Germany).

2.3. ISYPT Extraction to Produce a Sample

To prepare an ISYPT water extract, the constituent medicinal herbs were mixed in the weight ratio (g, *w/w*) shown in Table S1, and then 50 L of water was added for extraction at 100 °C for 2 h. Then, the solution of extract was freeze-dried to produce a powdered sample (988.8 g). The ISYPT sample thus prepared was stored at -20 °C until use.

2.4. Preparation of Sample Solution and Standard Stock Solution

Quantification for quality control of the ISYPT using the marker compounds (MBRSA, LQRTA, LQRTS, NRG, DDM, PCR, GCRZA, and SZDRA) was performed by HPLC–PDA. As a sample, 100 mg of lyophilized ISYPT was placed in a 10-mL volumetric flask, and DW and 70% MeOH were added, respectively. Then, each mixture was sonicated for 60 min and filtered using a 0.2 μ m polypropylene membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) before analysis.

A stock solution was prepared using each authentic standard at 1000 μ g/mL in MeOH and then stored at -4 °C until use.

2.5. HPLC Analysis for ISYPT Quality Assessment

Simultaneous analysis of ISYPT constituents was conducted using previous analytical protocols [20]. In brief, the HPLC system used in this analysis was a Shimadzu Prominence LC-20A series (Kyoto, Japan), and PDA was used to detect the constituents.

LCSolution software (version 1.24, SP1) was used to acquire and process data. Detailed parameters for HPLC for the quantitative analyses are shown in Table S2.

2.6. System Suitability

To develop a method for simultaneous analysis of marker compounds, system suitability factors, capacity factor (k'), relative retention (α), resolution (R_s), number of theoretical plates (N), and tailing factor (T_f), were identified.

2.7. Validation of the HPLC Analytical Method

Validation (linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision) of the HPLC method developed to assess the quality of ISYPT samples was conducted in the same manner as in a previous study [20] and according to the International Conference on Harmonisation guidelines [21].

3. Results and Discussion

3.1. Identification of the Major Constituents of Medicinal Herbs

To select the marker compounds to be quantified simultaneously for quality control of ISYPT, we identified the major active compounds in each medicinal herb: saikosaponin A (*B. falcatum*); mulberroside A (*M. alba*); polyporenic acid C and pachymic acid (*P. cocos*); schizandrol A, gomisins A, and gomisins N (*S. chinensis*); peimine and peiminine (*F. thunbergii*); amygdalin (*P. armeniaca*); naringin, didymin, and poncirin (*P. trifoliata*); platycodin D and platycodin D2 (*P. grandiflorum*); ginsenoside Rb1 and ginsenoside Rg1 (*P. ginseng*); no ingredient (*E. asinus*); liquiritin apioside, liquiritoside, liquiritigenin, and glycyrrhizic acid (*G. uralensis*); 6-gingerol (*Z. officinale*); and spinosin (*Z. jujuba*). Figure S2 shows the results confirming the major ingredients of each medicinal herb using TFA-containing DW-ACN as the mobile phase in the HPLC-PDA system.

3.2. Selection of Compounds to Evaluate the Quality of ISYPT

We detected eight marker compounds after HPLC of ISYPT samples containing the 23 constituents identified in Section 3.1 (Figure S3). After determining eight marker compounds for ISYPT (Figure S1), further studies such as establishing of simultaneous analysis method, method validation, and quantification were conducted.

3.3. Optimization of HPLC Analysis Conditions

Various parameters such as the manufacturer of the column (Phenomenex, Waters, Shiseido, PerkinElmer, and Younglin Biochrom), the column temperature (30 °C, 35 °C, 40 °C, and 45 °C), and the type of acid (formic acid, TFA, phosphoric acid, and acetic acid) were compared for simultaneous analysis of authentic standards for the eight marker compounds selected (MBRSA, LQRTA, LQRTS, NRG, DDM, PCR, GCRZA, and SZDRA). The HPLC analysis conditions ultimately selected were as follows: Gemini C18 analytical column (internal diameter D. 4.6 mm $\times$ length 250 mm, particle size 5 μ m, Phenomenex, Torrance, CA, USA), column temperature of 35 °C, and a DW-ACN system, both components containing 0.1% (*v/v*) TFA, as the mobile phase. All authentic standards were detected within 40 min and retention times of the standards, MBRSA, LQRTA, LQRTS, NRG, DDM, PCR, GCRZA, and SZDRA, were 13.45, 19.73, 20.12, 21.79, 26.37, 27.03, 34.33, and 38.81 min, respectively. A typical HPLC chromatogram is presented in Figure 1.

3.4. Validation of the HPLC Analytical Method

To analyze the stability of the HPLC method, system suitability for parameters such as k' , α , N , R_s , and T_f was tested using a solution of authentic standards. As shown in Table S3, as a result of considering all parameters, the method is suitable for simultaneous analysis of the chosen standards. The coefficients of determination (r^2), which are indicators of linearity, were all 0.9999, showing good linearity (Table 1). The LOD 0.03–0.41 μ g/mL and LOQ 0.08–1.21 μ g/mL (Table 1) were calculated in the same way as they were in previous studies [15,16]. As shown in Table 2, the extraction recovery test performed to verify accuracy was 95.67–102.38% in samples to which authentic standards were added at various concentrations (low, medium, and high) to 103.50%, and relative standard

deviation (RSD) (%) was 0.23–2.35%. Repeatability was expressed as RSD (%) after six repeated measurements using a solution of the standards. All authentic standards had <1.0% RSD for retention time and peak area, showing good repeatability (Table 3 and Tables S4 and S5). The intra-(one day) and interday (three consecutive days) precision also showed good results with RSD (%) <4.0% (Table 3). Based on verification results indicated above, the method for quality control of ISYPT developed in the present study is acceptable.

Figure 1. Typical HPLC chromatograms of (A) authentic standards, (B) ISYPT DW extract, and (C) ISYPT 70% MeOH extract of ISYPT samples at 250, 275, 280, and 325 nm. MBRSA (1), LQRTA (2), LQRTS (3), NRG (4), DDM (5), PCR (6), GCRZA (7), and SZDRA (8).

Table 1. Linear range, regression equation, coefficient of determination (r^2), limits of detection (LOD), and quantification (LOQ) for the simultaneous analysis of eight authentic standards by HPLC ($n = 3$).

Analyte	Linear Range (µg/mL)	Regression Equation ^a $y=ax+b$	r^2	LOD ^b (µg/mL)	LOQ ^c (µg/mL)
MBRSA	1.56–100.00	$y = 29,664.63x - 213.40$	0.9999	0.40	1.21
LQRTA	0.78–50.00	$y = 14,626.08x + 664.05$	0.9999	0.25	0.77
LQRTS	0.31–20.00	$y = 23,686.70x + 387.65$	0.9999	0.10	0.30
NRG	0.78–50.00	$y = 17,632.59x + 1673.16$	0.9999	0.11	0.33
DDM	0.47–30.00	$y = 19,190.72x + 964.97$	0.9999	0.05	0.16
PCR	1.56–100.00	$y = 18,209.46x + 2968.69$	0.9999	0.41	1.23
GCRZA	0.78–50.00	$y = 9833.62x + 170.54$	0.9999	0.25	0.74
SZDRA	0.31–20.00	$y = 20,364.60x + 114.65$	0.9999	0.03	0.08

^a y is the mean peak area and x is the concentration of each analyte. ^b $LOD = 3.3 \times \sigma/S$ and ^c $LOQ = 10 \times \sigma/S$ (where σ is the standard deviation of the y -intercept and S is the slope of the calibration curve).

Table 2. Extraction recovery (%) test for the analytical method using eight authentic standards ($n = 5$).

Analyte	Spiked Conc. (µg/mL)	Found Conc. (µg/mL)	Recovery (%) ^a	SD	RSD (%) ^b
MBRSA	6.00	5.81	96.87	1.28	1.33
	15.00	14.44	96.26	2.08	2.16
	30.00	29.75	99.18	1.32	1.33
LQRTA	2.00	1.96	97.87	2.30	2.35
	5.00	5.12	102.38	1.93	1.89
	10.00	9.65	96.47	1.57	1.63
LQRTS	1.00	0.99	99.09	1.80	1.82
	2.00	1.97	98.52	2.08	2.11
	4.00	4.06	101.60	1.52	1.50
NRG	4.00	4.09	102.15	1.97	1.93
	10.00	9.90	99.05	1.54	1.55
	20.00	19.23	96.15	0.93	0.97
DDM	1.00	0.98	98.02	1.92	1.96
	2.00	2.03	101.60	1.44	1.42
	4.00	3.97	99.24	0.66	0.66
PCR	6.00	6.10	102.17	1.11	1.08
	15.00	15.00	99.98	0.80	0.80
	30.00	29.16	97.15	0.22	0.23
GCRZA	2.00	2.03	101.69	1.00	0.99
	5.00	4.98	99.58	1.34	1.35
	10.00	9.62	96.17	0.69	0.72
SZDRA	1.00	0.97	97.00	1.74	1.80
	2.00	1.91	95.67	0.67	0.70
	4.00	3.94	98.51	0.61	0.62

^a Recovery (%) = found concentration/spiked concentration $\times$ 100%. ^b RSD (%) = standard deviation (SD)/mean $\times$ 100%.

3.5. Simultaneous Quantification of the Eight Marker Constituents of ISYPT

The HPLC method newly developed and verified in the present study was applied to the simultaneous analysis of eight marker constituents in lyophilized ISYPT. The constituents were quantified using a full scan from 190 to 400 nm with PDA detector, and each constituent was quantified based on its maximum UV absorption as follows: 250 nm for GCRZA and SZDRA, 275 nm for LQRTA and LQRTS, 280 nm for NRG, DDM, and PCR, and 325 nm for MBRSA. The eight representative marker constituents (MBRSA, LQRTA,

LQRTS, NRG, DDM, PCR, GCRZA, and SZDRA) were detected in the lyophilized ISYPT samples at concentrations ranging 0.09–5.95 mg/g (Table 4).

Table 3. Precision of the assay of the eight authentic standards.

Analyte	Conc. (µg/mL)	Intraday (n = 5)			Interday (n = 5)			Repeatability (n = 6)	
		Found Conc. (µg/mL)	Precision (RSD %) ^a	Accuracy (%)	Found Conc. (µg/mL)	Precision (RSD %)	Accuracy (%)	RSD (%) of Retention Time	RSD (%) of Peak Area
MBRSA	25.00	25.22	0.68	100.87	26.33	3.72	105.30	0.04	0.72
	50.00	50.94	1.60	101.88	52.77	3.20	105.55		
	100.00	105.58	1.75	105.58	107.74	2.10	107.74		
LQRTA	12.50	12.63	0.74	101.06	13.19	3.77	105.54	0.02	0.69
	25.00	25.50	2.07	102.01	26.40	3.16	105.60		
	50.00	52.81	2.03	105.62	53.77	2.10	107.54		
LQRTS	5.00	5.05	0.72	101.08	5.29	3.89	105.75	0.02	0.71
	10.00	10.21	2.00	102.10	10.57	3.15	105.73		
	20.00	21.14	2.07	105.69	21.52	2.13	107.60		
NRG	12.50	12.66	0.74	101.31	13.25	3.97	106.02	0.02	0.71
	25.00	25.59	1.86	102.37	26.53	3.19	106.11		
	50.00	52.92	1.90	105.83	53.96	2.10	107.92		
DDM	7.50	7.60	0.71	101.37	7.93	3.73	105.79	0.01	0.89
	15.00	15.29	0.99	101.96	15.90	3.26	106.00		
	30.00	31.51	1.73	105.05	32.29	2.28	107.64		
PCR	25.00	25.33	0.67	101.30	26.63	3.86	106.50	0.01	0.76
	50.00	51.20	1.84	102.39	53.03	3.14	106.05		
	100.00	105.71	1.94	105.71	107.74	2.06	107.74		
GCRZA	12.50	12.61	0.76	100.92	13.13	3.51	105.05	0.01	0.72
	25.00	25.40	1.97	101.60	26.27	3.12	105.10		
	50.00	52.58	1.94	105.17	53.56	2.11	107.12		
SZDRA	5.00	5.07	0.68	101.39	5.31	3.96	106.14	0.01	0.71
	10.00	10.22	1.90	102.16	10.57	3.13	105.68		
	20.00	20.84	1.31	104.20	21.38	2.36	106.88		

^a RSD (%) = standard deviation (SD)/mean × 100%.

Table 4. Concentrations of eight representative marker constituents in ISYPT samples by HPLC–PDA (n = 3).

Constituent	Concentration (mg/g)			
	DW Solution		70% MeOH Solution	
	Mean ± SD (×10 ^{−1})	RSD (%)	Mean ± SD (×10 ^{−1})	RSD (%)
MBRSA	5.29 ± 0.24	0.45	5.95 ± 0.06	0.10
LQRTA	1.86 ± 0.06	0.31	1.97 ± 0.14	0.70
LQRT	0.34 ± 0.03	0.80	0.34 ± 0.02	0.65
NRG	3.06 ± 0.10	0.33	3.33 ± 0.29	0.87
DDM	0.60 ± 0.03	0.58	0.66 ± 0.01	0.14
PCR	4.23 ± 0.14	0.32	4.84 ± 0.04	0.08
GCRZA	1.51 ± 0.01	0.09	1.80 ± 0.05	0.27
SZDRA	0.09 ± 0.01	1.20	0.11 ± 0.01	1.27

4. Conclusions

In the present study, a method of analysis for quality control of ISYPT was developed using a HPLC–PDA system. The method for simultaneous analysis of representative marker constituents was verified using various parameters, including linearity, LOD, LOQ, accuracy, and precision. This analytical method can be used to characterize the herbal medicine prescription for basic research.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11114819/s1>, Figure S1: Chemical structures of eight representative marker constituents of ISYPT; Figure S2: HPLC chromatograms of constituents of the herbal medicine formula identifying its major components. (a): *B. falcatum*; (b): *M. alba*; (c): *P. cocos*; (d): *S. chinensis*; (e): *F. thunbergii*; (f): *P. armeniaca*; (g): *P. trifoliata*; (h): *P. grandiflorum*; (i): *P. ginseng*; (j): *E. asinus*; (k): *G. uralensis*; (l): *Z. officinale*; and (m): *Z. jujuba*; Figure S3: HPLC chromatograms of the standard solution (a) DW extract (b), and 70% MeOH extract (c) of ISYPT samples. Mulberroside A (1), amygdalin (2), spinosin (3), liquiritin apioside (4), liquiritoside (5), naringin (6), peimine (7), peiminine (8), ginsenoside Rg1 (9), platycodin D2 (10), didymin (11), liquiritigenin (12), platycodin D (13), poncirin (14), ginsenoside Rb1 (15), glycyrrhizic acid (16), saikosaponin A (17), 6-gingerol (18), schizandrol A (19), gomisin A (20), polyporenic acid C (21), gomisin N (22), and pachymic acid (23); Table S1: Information about and composition of ISYPT; Table S2: HPLC parameters for quantification of ISYPT constituents; Table S3: System suitability for authentic standards of the eight marker compounds in the analytical method using HPLC–PDA; Table S4: Repeatability of HPLC retention time for the eight authentic standards ($n = 6$); Table S5: Repeatability of HPLC peak area for the eight authentic standards ($n = 6$).

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