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Quality Assessment of Insamyangpye Decoction by Liquid Chromatography Tandem Mass Spectrometry Multiple Reaction Monitoring

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Abstract: Insamyangpye decoction (ISYPD) is an oriental herbal prescription used in Korea to treat lung-related diseases such as chronic obstructive pulmonary disease. ISYPD is a complex prescription consisting of 13 herbal medicines, and ISYPD sample was obtained by adding 50 L of distilled water to a mixture (5 kg) of 13 herbal medicines, extracting at 100 °C for 2 h using an electric extractor, and freeze-drying. In this study, an accurate and sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) method based on multiple reaction monitoring (MRM) was developed and verified for quality assessment of ISYPD using 10 marker components: mulberroside A (**1**), amygdalin (**2**), liquiritin apioside (**3**), naringin (**4**), poncirin (**5**), platycodin D (**6**), ginsenoside Rb1 (**7**), glycyrrhizin (**8**), saikosaponin A (**9**), and schizandrin (**10**). These marker compounds were separated using an Acquity UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) maintained at 30 °C with a mobile phase elution gradient of acetonitrile in distilled water, both containing 0.1% (*v*/*v*) trifluoroacetic acid. Marker components were quantified using the LC–MS/MS MRM method developed and validated, and found at 0.09–7.47 mg/g.

Keywords: quality assessment; insamyangpye decoction; liquid chromatography tandem mass spectrometry

Citation: Seo, C.-S.; Lee, M.-Y. Quality Assessment of Insamyangpye Decoction by Liquid Chromatography Tandem Mass Spectrometry Multiple Reaction Monitoring. *Processes* **2021**, *9*, 831. https://doi.org/10.3390/pr9050831

Academic Editor: Małgorzata Dołowy

Received: 13 April 2021
Accepted: 7 May 2021
Published: 10 May 2021

1. Introduction

Standardization is essential for quality consistency of traditional Korean medicines (TKM), traditional Chinese medicines, and Kampo medicines. These medicines are difficult to standardize because their formulas are composed of various kinds of herbal medicines and numerous components. For their standardization, equipment for analysis such as high-performance liquid chromatography combined with a photo-diode array (HPLC–PDA) detector, liquid chromatography tandem mass spectrometry (LC–MS/MS), gas chromatography (GC), and gas chromatography–mass spectrometry (GC–MS) have been widely used [1–5]. In particular, LC–MS/MS, which can produce a sensitive, accurate, and quick analysis, is currently one of the most widely used type of analytical system.

Insamyangpye decoction (ISYPD) is composed of 13 herbal medicines (Table S1). ISYPF is a TKM used to treat cough, phlegm, and fever caused by lung diseases and is listed in Heo Jun's Donguibogam [6]. However, no studies have been conducted to determine the efficacy of its biological activity. As major components of 13 raw materials, triterpenoids, stilbenoids, flavonoids, lignans, alkaloids, phenols, and amino acids have been reported [7–18]. In addition, studies analyzed using HPLC or LC–MS for each herbal medicine constituting ISYPD have been reported [7–15]. These studies focus on the qualitative and quantitative analysis of constituent herbal medicines, but ISYPD is a complex prescription that combines all 13 herbal medicines, and a simultaneous analysis method for quality assessment of ISYPD has not yet been reported.

In this study, we focused on the development of a simultaneous analysis method for efficient quality control using 10 marker compounds, mulberroside A (**1**), amygdalin (**2**), liquiritin apioside (**3**) naringin (**4**), poncirin (**5**), platycodin D (**6**), ginsenoside Rb1 (**7**), glycyrrhizin (**8**), saikosaponin A (**9**), and schizandrin (**10**), of ISYPD samples using LC–MS/MS coupled with an electrospray ionization (ESI) interface in multiple reaction monitoring (MRM) mode.

2. Materials and Methods

2.1. Plant Materials

In March 2018, the 13 types of medicinal herbs that make up ISYPD were obtained from a specialized supplier of herbal medicines, Kwangmyungdang Medicinal Herbs (KMH; Ulsan, Korea). Each raw herb was identified morphologically by Dr. Seung-Yeol Oh, pharmacognosist, (CEO of KMH) according to the guidelines of the Korean Ministry of Food and Drug Safety [19]. Specimens of each (2018CA03–1 to 2018CA03–13) have been deposited in the herbarium Korea Institute of Oriental Medicine.

2.2. Chemicals and Reagents

Authentic reference standards (Figure S1) for LC–MS/MS quantitative analysis of ISYPD samples were supplied by manufacturers of phytochemical standards: compound **1** ($C_{26}H_{32}O_{14}$, 98.1%) from Ensol Biosciences (Daejeon, Korea); compounds **2** ($C_{20}H_{27}NO_{11}$, $\geq$99.0%) and **4** ($C_{27}H_{32}O_{14}$, 95.0%) from Merck (Seoul, Korea); compounds **3** ($C_{26}H_{30}O_{13}$, $\geq$98.0%), **5** ($C_{28}H_{34}O_{14}$, 98.9%), and **7** ($C_{54}H_{92}O_{23}$, 98.5%) from Shanghai Sunny Biotech Co. (Shanghai, China); compounds **8** ($C_{42}H_{62}O_{16}$, 99.4%) and **9** ($C_{42}H_{68}O_{13}$, 99.4%) from Fujifilm Wako Pure Chemicals (Osaka, Japan); compounds **6** ($C_{57}H_{92}O_{28}$, 99.9%) and **10** ($C_{24}H_{32}O_{7}$, 99.3%) from ChemFaces Biochemical Co. (Wuhan, China) and Chengdu Biopurify Phytochemicals (Chengdu, China), respectively. LC–MS-grade solvents (methanol, acetonitrile, and distilled water) were purchased from ThermoFisher Scientific (Seoul, Korea) and LC–MS-grade reagent (trifluoroacetic acid; TFA, $\geq$99.0%) from Merck (Seoul, Korea).

2.3. Preparation of ISYPD Extract

ISYPD was produced from the formulation of 13 raw medicinal herbs, Bupleuri Radix (138.06 g), Mori Radicis Cortex (519.03 g), Poria Sclerotium (363.32 g), Schisandrae Fructus (363.32 g), Fritillariae Thunbergii Bulbus (363.32 g), Armeniacae Semen (363.32 g), Ponciri Fructus Immaturus (363.32 g), Platycodonis Radix (363.32 g), Ginseng Radix (259.52 g), Asini Corii Colla (259.52 g), Glycyrrhizae Radix et Rhizoma (259.52 g), Zingiberis Rhizoma Recens (207.61 g), and Zizyphi Fructus (276.82 g) and freeze-dried to make a powder sample. The details of the extraction method are presented in published protocols [1]. The lyophilized extract showed a yield of 19.78% (988.8 g).

2.4. Preparation of Samples and Standard Solutions for the Quantitative Analysis

To analyze the main marker components in the ISYPD sample using LC–MS/MS, 10.0 mg of the powdered ISYPD sample was added to a 100-mL volumetric flask filled with 50% methanol. The mixed solution was continuously subjected to ultrasonic extraction and vortexing for 10.0 min each. The extract was filtered through a 0.2 μm membrane filter and used for the analysis.

A solution of an authentic standard for each marker component used in this study was prepared at a concentration of 100.00 μg/mL using methanol, and then stored in a refrigerator (approximately 4 °C) and used for further analysis.

2.5. Instrument and Operating Conditions for Simultaneous Analysis of Compounds **1–10** in the ISYPD Sample

The instruments and methods for LC–MS/MS simultaneous quantification of compounds **1–10** in the ISYPD sample were applied to this study by modifying a previously published protocol [5]. Briefly, a simultaneous analysis of major markers was conducted using an Acquity UPLC H-Class system (Waters, Milford, MA, USA), consisting of a quaternary solvent manager and sample manager, with a Xevo TQ-S micro triple quadrupole MS system (Waters, Milford, MA, USA). Detailed operating conditions for UPLC and MS are presented in Table 1. The ionization mode, MRM transition (precursor ion to product ion), cone voltage, and collision energy for LC–MS/MS MRM analysis of compounds **1–10** are summarized in Table 2.

Table 1. LC-MS/MS MRM(Liquid chromatography tanderm mass spectrometry multiple reaction monitoring) transitions for quantification of compounds **1–10**.

UPLC Conditions				**MS (Mass Spectrometry) Conditions**	
UPLC (Ultra performance liquid chromatography) system	Acquity UPLC H-Class			MS system	Xevo TQ-S micro
Column	Acquity UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm)			MS software	MassLynx (version 4.2)
Column temp.	30 °C			Ionization mode	ESI^+ (Electrospray ionization positive)/ESI^- (Electrospray ionization nagative)
Sample temp.	20 °C			Acquisition mode	MRM (multiple reaction monitoring)
Injection volume	2.0 μL			Capillary voltage	3.3 V
Flow rate	0.3 mL/min			Cone gas flow	80 L/h
Mobile phase A	Distilled water with 0.1% TFA (trifluoroacetic acid)			Desolvation temp.	300 °C
Mobile phase B	Acetonitrile with 0.1% TFA			Desolvation gas flow	300 L/h
Gradient	Time (min)	A (%)	B (%)	Source temp.	150 °C
	0.00	95	5		
	11.43	40	60		
	14.29	5	95		
	15.71	5	95		
	17.14	95	5		
	20.00	95	5		

Table 2. LC–MS/MS MRM transitions for quantification of compounds **1–10**.

Markers	Ion Mode	Molecular Weight	MRM Transition	Cone Voltage (V)	Collision Energy (eV)	Retention Time (min)
1	Positive	568.18	569.21→244.99	16	18	2.40
2	Positive	457.16	480.14→347.01	96	26	2.84
3	Negative	550.17	549.15→118.94	76	48	4.16
4	Positive	580.18	581.18→152.94	18	44	4.83
5	Positive	594.19	595.21→286.98	6	20	6.23
6	Positive	1224.58	1225.66→485.23	14	46	6.57
7	Positive	1108.6	1109.7→162.94	28	44	7.57
8	Positive	822.4	823.46→453.2	18	20	8.48
9	Positive	780.47	781.51→437.2	14	16	9.09
10	Positive	432.21	433.22→346.01	26	24	9.25

2.6. Validation of the LC–MS/MS MRM Method

The LC–MS/MS MRM method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy (recovery), and precision according to the International Conference on Harmonization guidelines and previous methods [2,20].

Briefly, the linearity was determined by the coefficient of determination (r^2) of the calibration curve prepared for various concentrations, and the LOD and LOQ were calculated from the following Equations (1) and (2) using the calibration curve data.

$$\text{LOD} = 3.3 \times \sigma/S \tag{1}$$

$$\text{LOQ} = 10 \times \sigma/S \tag{2}$$

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

Accuracy was determined using a recovery test by adding authentic standards to various concentrations. Briefly, known standard concentrations (low, medium, and high) were added to 10 mg of each ISYPD sample, adjusted to 100 mL with 50% methanol, and extracted according to the extraction protocol in Section 2.4. Precision was determined using the relative standard deviation (RSD) for repeatability (intraday, within one day), intermediate precision (interday, 3 consecutive days), and reproducibility.

3. Results and Discussion

3.1. Development of the LC–MS/MS MRM Method

In the development of the LC–MS/MS MRM method for ISYPD samples, the UPLC conditions for simultaneous analysis of compounds **1–10** were established as follows: an Acquity UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 µm), a column temperature of 30 °C, and a mobile phase gradient of acetonitrile in distilled water, both 0.1% (v/v) TFA (Table 1). In addition, the MS system was optimized for various parameters as shown in Table 2. The LC–MS/MS MRM method was applied to the ISYPD samples to elute all marker analytes within 10 min (Figure 1).

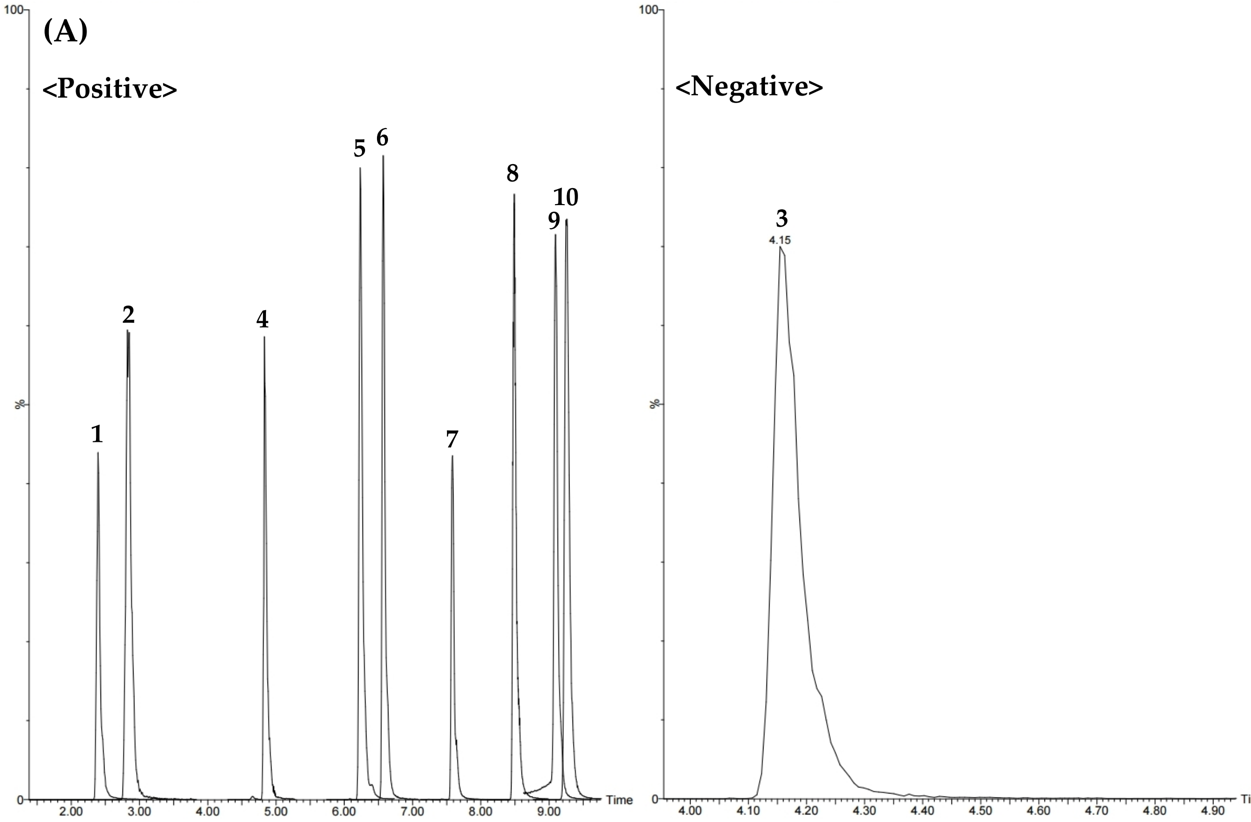

Figure 1. *Cont.*

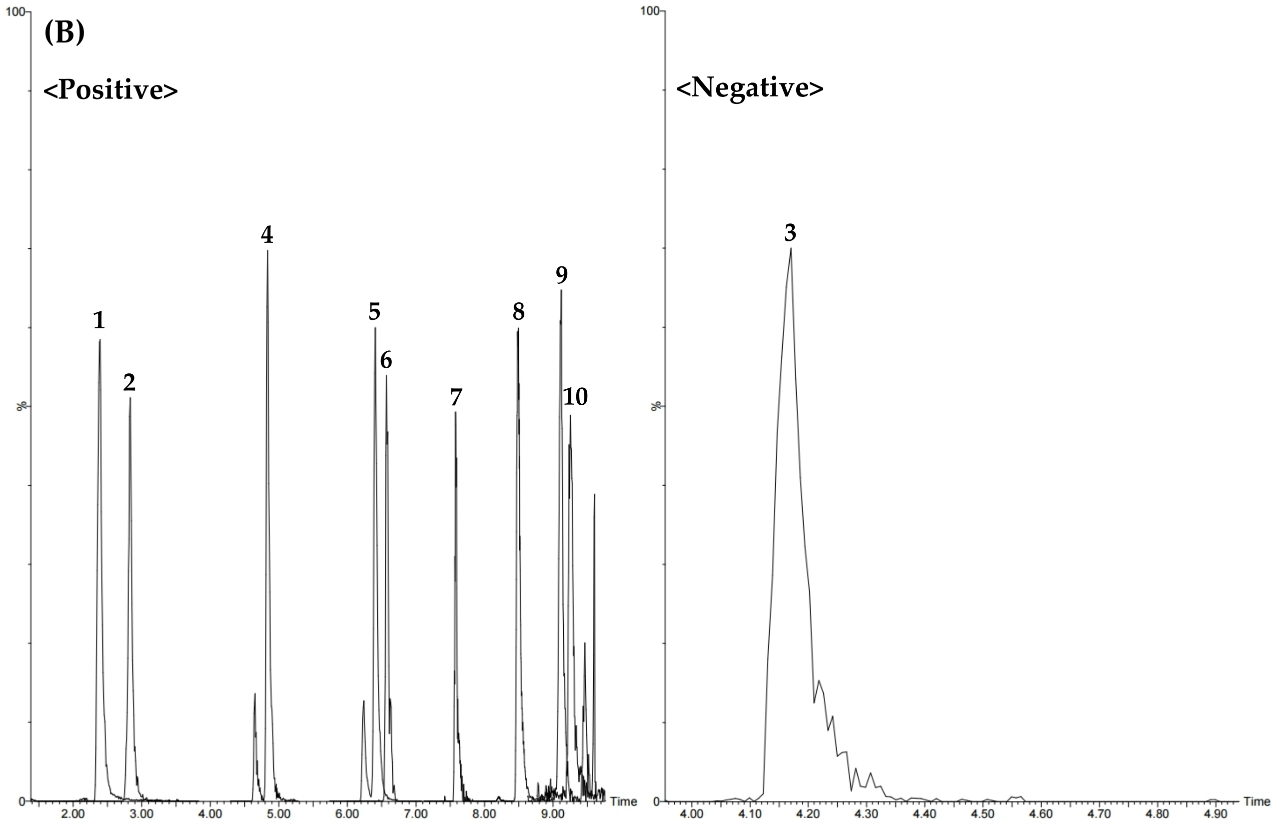

Figure 1. Total ion chromatograms of mixtures of compounds **1**–**10** (**A**) and 50% methanol extract of the freeze-dried ISYPD sample (**B**) measured by LC–MS/MS MRM in positive and negative ion modes. Mulberroside A (**1**), amygdalin (**2**), liquiritin apioside (**3**), naringin (**4**), poncirin (**5**), platycodin D (**6**), ginsenoside Rb1 (**7**), glycyrrhizin (**8**), saikosaponin A (**9**), and schizandrin (**10**).

3.2. MRM Transition of Each Marker Compound in the LC–MS/MS Simultaneous Analysis

In the MS spectra of the 10 marker analytes measured using the positive/negative ESI sources, compounds **1**, **2**, and **4**–**10** were detected in the positive ion mode and compound **3** was detected in negative ion mode, respectively (Figure 1, Table 2). For LC–MS/MS simultaneous analysis, MRM transition conditions for each marker analyte were determined. In the optimized LC–MS/MS system, the precursor ion (Q1) peak of compound **1** was detected at *m*/*z* 569.21, and a product ion (Q3) peak was detected at *m*/*z* 244.99 in the form of $[M + H - 2Glc]^+$ in which two glucopyranosyl groups were released from the Q1 peak [21]. Compound **2** was observed in the form of $[M + Na - aglycone]^+$ at *m*/*z* 347.01 (Q3) in which the aglycone was eliminated from the Q1 peak [22]. Compounds **3** and **4** produced Q3 peaks formed by a retro-Diels-Alder reaction of the aglycone of each compound at *m*/*z* 118.94 and *m*/*z* 152.94 [23,24]. In compound **5**, *m*/*z* 286.98 $[M + H - Glc - Rham]^+$ formed by removing glucose and rhamnose from the *m*/*z* 595.21 (Q1) peak was set as the Q3 peak [25]. The Q3 peaks of compounds **6** and **8** were observed as 485.23 and 453.20 in the form of $[M + H - Api - Xyl - Rha - Ara - CH_2O_4]^+$ and $[M + H - 2GalA - H_2O]^+$ that lost 740 Da and 370 Da at each Q1 peak [23,26]. Compounds **9** and **10** were detected as Q3 peaks at *m*/*z* 437.20 ($[M + H - Glc - Fuc - 2H_2O]^+$) and *m*/*z* 346.01 ($[M + H - C_5H_{10}O]^+$), respectively; these Q3 peaks were formed by the elimination of Glc − Fuc − $2H_2O$ and two $C_5H_{10}O$ molecules from each Q1 peak [27,28]. Compound **8** was observed as a Q3 peak at *m*/*z* 162.94 $[Glc + H - H_2O]^+$, in which one water molecule was missing from glucose [29].

3.3. Verification of the LC–MS/MS Method

The LC–MS/MS method was validated for specificity, linearity, LOD, LOQ, accuracy (recovery), and precision. As shown in Figure S2, the specificity was confirmed as compounds were well separated without interference from each other when the retention times of all components were compared. The calibration curve showed the r^2 values of compounds **1**–**10** were between 0.9914 to 0.9997, showing good linearity, and the LOD and LOQ were calculated as 0.002–39.093 µg/L and 0.005–130.310 µg/L, respectively (Table 3). Recovery was 90.00–114.97% with RSD less than 20% and is shown in Table 4. The RSD values for the reproducibility of all marker analytes were measured as 0.21–0.90% for the retention time and 2.48–14.66% for the peak area. In addition, the RSD values of intraday, interday, and accuracy did not exceed 20% for any marker (Table 5). These verification results show that the LC–MS/MS MRM method developed is suitable for simultaneous analysis of marker compounds to assess the quality of the ISYPD samples.

Table 3. Linear range, regression equation, coefficient of determination (r^2), limit of detection (LOD), and limit of quantification (LOQ) for LC–MS/MS MRM analysis of compounds **1**–**10**.

Marker	Linear Range (µg/L)	Regression Equation [a] $y = ax + b$	r^2	LOD (µg/L)	LOQ (µg/L)
1	200.00–3200.00	$y = 16.61x - 30.28$	0.9996	3.657	12.190
2	200.00–3200.00	$y = 2.34x + 31.68$	0.9983	39.093	130.310
3	75.00–1200.00	$y = 1.06x - 14.60$	0.9976	6.009	20.030
4	125.00–2000.00	$y = 18.62x + 59.88$	0.9952	1.140	3.800
5	25.00–400.00	$y = 33.51x + 70.79$	0.9996	2.151	7.170
6	7.50–120.00	$y = 3.45x + 2.65$	0.9914	2.094	6.980
7	2.50–40.00	$y = 10.00x - 3.38$	0.9989	0.002	0.005
8	75.00–1200.00	$y = 66.65x - 332.05$	0.9997	6.939	23.130
9	7.50–120.00	$y = 23.00x + 3.17$	0.9989	1.365	4.550
10	2.5.00–40.00	$y = 85.14x + 3.56$	0.9986	0.123	0.410

[a] x and y mean peak area and concentration of each compound, respectively.

Table 4. Extract recovery tests of compounds **1**–**10** in the developed LC–MS/MS MRM method.

Marker	Spiked Amount (µg/L)	Found Amount (µg/L)	Recovery (%) [a]	SD (Standard Deviation)	RSD (%) (Relative Standard Deviation (%))
1	100.00	90.00	90.00	6.00	6.62
	200.00	229.93	114.97	5.00	4.34
	400.00	417.20	104.30	2.30	2.25
2	100.00	94.17	94.17	7.00	7.42
	200.00	198.57	99.29	8.90	8.98
	400.00	428.97	107.24	6.10	5.67
3	37.50	38.63	103.01	18.40	17.86
	75.00	78.77	105.03	12.40	11.84
	150.00	165.60	110.40	4.20	3.78
4	62.50	65.50	104.80	7.00	6.64
	125.00	119.23	95.38	10.60	11.14
	250.00	263.23	105.29	8.40	7.97
5	12.50	13.20	105.60	2.10	2.00
	25.00	25.90	103.60	4.20	4.09
	50.00	53.53	107.06	6.50	6.04
6	3.75	3.40	90.67	11.60	12.82
	7.50	8.33	111.07	5.40	4.85
	15.00	16.27	108.47	8.40	7.78
7	1.25	1.30	104.00	8.00	7.69
	2.50	2.57	102.80	14.00	13.68
	5.00	4.67	93.40	17.90	19.21

Table 4. *Cont.*

Marker	Spiked Amount (μg/L)	Found Amount (μg/L)	Recovery (%) [a]	SD (Standard Deviation)	RSD (%) (Relative Standard Deviation (%))
8	37.50	40.27	107.39	5.40	4.99
	75.00	74.50	99.33	3.30	3.30
	150.00	152.80	101.87	1.00	1.02
9	3.75	3.57	95.20	8.10	8.57
	7.50	7.97	106.27	5.40	5.07
	15.00	14.07	93.80	11.70	12.52
10	1.25	1.20	96.00	8.00	8.33
	2.50	2.77	110.80	6.10	5.52
	5.00	5.43	108.60	11.40	10.47

[a] Recovery (%) = found amount/spiked amount × 100.

Table 5. Precision and accuracy verification of compounds **1**–**10** in the developed LC–MS/MS MRM method.

Markers	Conc. (μg/L)	Intraday (*n* = 5)			Interday (*n* = 5)		
		Observed Conc. (Concentration) (μg/L)	Precision (%) [a]	Accuracy (%)	Observed Conc. (μg/L)	Precision (%)	Accuracy (%)
1	100.00	90.00	6.62	90.00	106.37	7.85	106.40
	200.00	229.93	4.34	115.00	219.26	5.08	109.60
	400.00	417.20	2.25	104.30	371.90	11.46	93.00
2	100.00	94.17	7.42	94.20	100.77	12.02	100.80
	200.00	198.57	8.98	99.30	200.98	17.83	100.50
	400.00	428.97	5.67	107.20	392.52	13.05	98.10
3	37.50	38.63	17.86	103.00	37.02	16.75	98.70
	75.00	78.77	11.84	105.00	84.23	3.66	112.30
	150.00	165.60	3.78	110.40	151.03	15.01	100.70
4	62.50	65.50	6.64	104.80	65.67	11.03	105.10
	125.00	119.23	11.14	95.40	135.20	9.19	108.20
	250.00	263.23	7.97	105.30	262.77	13.83	105.10
5	12.50	13.20	2.00	105.60	13.23	3.49	105.90
	25.00	25.90	4.09	103.60	24.73	8.39	98.90
	50.00	53.53	6.04	107.10	48.17	16.48	96.30
6	3.75	3.40	12.82	90.70	3.90	5.13	104.00
	7.50	8.33	4.85	111.10	8.40	1.19	112.00
	15.00	16.27	7.78	108.40	14.67	14.05	97.80
7	1.25	1.30	7.69	104.00	1.28	10.25	102.40
	2.50	2.57	13.68	102.70	2.78	3.93	111.00
	5.00	4.67	19.21	93.30	4.96	15.15	99.10
8	37.50	40.27	4.99	107.40	37.51	2.76	100.00
	75.00	74.50	3.30	99.30	74.55	4.79	99.40
	150.00	152.80	1.02	101.90	148.97	5.63	99.30
9	3.75	3.57	8.57	95.10	3.91	8.03	104.20
	7.50	7.97	5.07	106.20	7.84	8.88	104.60
	15.00	14.07	12.52	93.80	15.08	8.92	100.50
10	1.25	1.20	8.33	96.00	1.17	9.90	93.30
	2.50	2.77	5.52	110.70	2.70	7.41	108.00
	5.00	5.43	10.47	108.70	5.33	8.86	106.70

[a] Precision is expressed as RSD (%) = (SD/mean) × 100.

3.4. Simultaneous Determination of Compounds **1–10** *in the ISYPD Samples*

In this study, we tried to analyze a total of 19 components such as saikosaponin A (Bupleuri Radix), mulberroside A (Mori Radicis Cortex), dehydropachymic acid, pachymic acid, and polyporenic acid C (Poria Sclerotium), gomisn A, gomisin N, and schizandrin (Schisandrae Fructus), peimine (Fritillariae Thunbergii Bulbus), amygdalin (Armeniacae Semen), naringin and poncirin (Ponciri Fructus Immaturus), platycodin D (Platycodonis Radix), ginsenoside Rb1 (Ginseng Radix), glycyrrhizin, liquiritin apioside, and liquiritin (Glycyrrhizae Radix et Rhizoma), 6-gingerol (Zingiberis rhizome Recens), and spinosin (Zizphi Fructus) for quality control of ISYPD by referring to the Chinese Pharmacopoeia and references [7–18]. As a result, only 10 components were detected in the ISYPD sample and were determined as marker compounds.

The marker compounds in the ISYPD samples were quantified using MRM mode as shown in Table 2, and compounds **1–10** were detected at 2.40, 2.84, 4.16, 4.83, 6.23, 6.57, 7.57, 8.48, 9.09, and 9.25 min, respectively (Figure 1 and Figure S2). Compounds **1–10** were detected in the range 0.09–7.47 mg/g using the LC–MS/MS analysis method developed and validated using the ISYPD sample (Table 6). Among these components, compounds **1** and **2**, which are maker compounds of *Morus alba* L. and *Prunus armeniaca* L., showed the highest levels at 7.47 mg/g and 6.12 mg/g, respectively (Table 6).

Table 6. Amount of compounds **1–10** in an ISYPD sample by the LC–MS/MS MRM method (*n* = 3).

Marker	Amount		
	Mean (mg/g)	**SD**	**RSD (%)**
1	7.47	0.33	4.35
2	6.12	0.25	4.05
3	1.72	0.09	5.09
4	3.86	0.18	4.76
5	1.08	0.12	10.71
6	0.37	0.04	11.09
7	0.09	0.01	11.84
8	2.69	0.02	0.60
9	0.36	0.01	1.79
10	0.10	0.00	1.15

4. Conclusions

The LC–MS/MS MRM method is an accurate and fast way to control the quality of ISYPD efficiently, and is validated for specificity, linearity, LOD, LOQ, accuracy (recovery), and precision. This method can be successfully applied to the simultaneous quantification of marker compounds in ISYPD and may be useful to generate basic data to standardize not only ISYPD, but also other traditional herbal prescriptions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9050831/s1, Figure S1. Chemical structures of 10 marker components of ISYPD, Figure S2. Extracted ion chromatograms of compounds **1–10** in the standard solution (A) and the ISYPD sample (B) by LC–MS/MS MRM mode. Mulberroside A (**1**), amygdalin (**2**), liquiritin apioside (**3**), naringin (**4**), poncirin (**5**), platycodin D (**6**), ginsenoside Rb1 (**7**), glycyrrhizin (**8**), saikosaponin A (**9**), and schizandrin (**10**), Table S1: Information and composition of ISYPD.

Author Contributions: Conceptualization, C.-S.S. and M.-Y.L.; performing experiments and analyzing data, C.-S.S.; writing—original draft preparation, C.-S.S.; funding acquisition, M.-Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Korea Institute of Oriental Medicine (grant No. KSN2013220).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article (tables and figures).

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

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