applied sciences

MDPI

Article

Simultaneous Quantification of Eight Marker Components in Traditional Herbal Formula, Haepyoyijin-Tang Using HPLC–PDA

Chang-Seob Seo * and Mee-Young Lee

Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon 34054, Korea; cozy11@kiom.re.kr

* Correspondence: csseo0914@kiom.re.kr; Tel.: +82-42-868-9361

Received: 13 May 2020; Accepted: 1 June 2020; Published: 3 June 2020

Abstract: Haepyoyijin-tang (HPYJT) is a traditional herbal prescription that is composed of 12 medicinal herbs. Although HPYJT is frequently used in patients with asthma in Korea, no quality assessment protocols have been developed. In the present study, qualitative and quantitative analyses were performed using high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry on the eight main components (mulberroside A, amygalin, liquiritin apioside, liquiritin, narirutin, hesperidin, rosmarinic acid, and glycyrrhizinic acid) to establish a quality control protocol for HPYJT. The simultaneous analysis method developed was satisfactorily validated with respect to linearity, limit of detection, limit of quantification, recovery, and precision. This analytical method thus provides an efficient approach for the evaluation of HPYJT quality.

Keywords: simultaneous quantification; Haepyoyijin-tang; HPLC–PDA

1. Introduction

Haepyoyijin-tang (HPYJT) is an herbal medicine prescription that consists of 12 medicinal herbs (Perillae Folium, Citri Unshius Pericarpium, Poria Sclerotium, Glycyrrhizae Radix et Rhizoma, Perillae Folium, Ephedrae Herba, Armeniaceae Semen, Mori Radicis Cortex, Asteris Radix et Rhizoma, Fritillariae Thunbergii Bulbus, Platycodonis Radix, and Zingiberis Rhizoma Recens) that is used in Korea to treat coughs and asthma caused by the common cold [1].

HPYJT has been demonstrated to exhibit biological action, such as protective effects on pulmonary injury caused by SO_2, and to affect airway mucin production and gene expression control in respiratory diseases that are accompanied by mucus hypersecretion [2,3]. The major components that make up HPYJT are phenolic compounds (e.g., homogentisic acid and 3,4-dihydroxybenzaldehyde) from Pinelliae Tuber [4], flavonoids (e.g., narirutin, hesperidin, and tamgeretom) from Citri Unshius Pericarpium [5], steroids (e.g., ergosterol peroxide), sesquiterpenoids (e.g., (*S*)-(+)-turmerone), and triterpenes (e.g., pachymic acid and polyporenic acid C) from Poria Sclerotium [6], flavonoids (e.g., liquiritin and liquiritin apioside) and triterpenes (e.g., glycyrrhizinic acid and 18β-glycyrrhizinic acid) from Glycyrrhizae Radix et Rhizoma [7], phenolic compounds (e.g., rosemary acid and elemicin), monoterpenes (e.g., perillaldehyde) from Perillae Folium [8], alkaloids (e.g., ephedrine and pseudoephedrine) form Ephedrae Herba [9], phenolic compounds (e.g., amygdalin and neoamygdalin) from Armeniacae Semen [10], phenolic compounds (e.g., mulberroside A) and flavonoids (e.g., kuwanon G and morusin) from Mori Radicis Cortex [11,12], phenylpropanoids (e.g., chlorogenic acid and 3,4-dicaffeoylqunic acid), flavonoids (e.g., quercetin and kampferol), and triterpenes (e.g., shionone) from Asteris Radix et Rhizoma [13,14], alkaloids (e.g., peimine and peiminine) from Fritillariae Thunbergii bulbus [15], triterpenes (e.g., platycodin D and platycoside E) from Platycodonis

Radix [16], and phenolic compounds (e.g., 6-gingerol and 6-shogaol) from Zingiberis Rhizoma Recens [17].

To establish an efficient quality control protocol for HPYJT, we developed and validated the first simultaneous quantitative analysis using high-performance liquid chromatography equipped with photodiode array detector (HPLC–PDA) system on the eight main constituents of HPYJT. Marker components for quality control of HPYJT were selected as follows: mulberroside A (MULA) from Mori Radicis Cortex, amygdalin (AMY) from Armeniacae Semen, liquiritin apioside (LIQA), liquiritin (LIQ), and glycyrrhizinic acid (GA) from Glycyrrhizae Radix et Rhizoma, hesperidin (HES) and narirutin (NAR) from Citri Unshius Pericarpium, and rosmarinic acid (RA) from Perillae Folium.

2. Materials and Methods

2.1. Plant Materials

Twelve raw herbal materials of HPYJT (Table S1) were provided from Kwangmyungdag Medicinal Herbs (Ulsan, Korea) in 2017. The origin of each herbal medicine was confirmed by pharmacognosist, Dr. Goya Choi, Herbal Medicine Resources Research Center (HMRRC), Korea Institute of Oriental Medicine (KIOM, Naju, Korea) in accordance with the guidelines for sensory test of herbal medicine [18]. Voucher specimens (2018-CA02-1 to 2018-CA02-12) were deposited at the KIOM.

2.2. Chemicals and Reagents

The eight constituents (Figure S1) used for the quality assessment of HPYJT sample were purchased from natural product suppliers: i.e., MULA (98.1%) from EnsolBioSciences (Daejeon, Korea); AMY (≥99.0%) and RA (97.0%) from Merck KGaA (Darmstadt, Germany); LIQA (≥98.0%) from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China); LIQ (99.6%), GA (99.1%), HES (98.6%), and NAR (99.5%) from Biopurify Phytochemicals Ltd. (Chengdu, China).

Methanol, acetonitrile, water, and formic acid (FA, 99.0%) were from J.T.Baker (Phillipsburg, NJ, USA) and FUJIFILM Wako Pure Chemical Co. (Osaka, Japan) as a solvent or reagent for HPLC analysis.

2.3. Preparation of HPYJT Water Decoction

The 12 herbal medicines constituting HPYJT were mixed at the weight ratio (g, w/w) shown in Table S1. These materials were formulated and extracted with distilled water using a COSMOS-660 electric extractor (Kyungseo E&P, Incheon, Korea) under the following conditions: extraction temperature, 100 °C; extraction time, 2 h; extraction pressure, 98 kPa. The water extract was filtered using the sieve (size 53 μm mesh) and freeze-dried to make a powder sample (703.9 g, 14.08% yield).

2.4. Preparations of Test Solution and Stock Solution

For quantitative determination of the eight analytes (MULA, AMY, LIQA, LIQ, NAR, HES, RA, and GA) in the HPYJT decoction sample, 100 mg of the freeze-dried HPYJT sample was dissolved in 10 mL of 70% methanol and then extracted using an ultrasonicator (Branson 8510; Denbury, CT, USA) for 60 min at room temperature. The extract was filtered through a 0.2-μm membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) before injection to HPLC system.

Each stock solution of eight constituents was made with methanol at a concentration of 1.0 mg/mL and kept refrigerated (approximately 4 °C).

2.5. HPLC–PDA Instruments and Analysis Conditions for the Phtochemical Determination

Phytochemical determination for quality evaluation of HPYJT was conducted by modifying previously reported analysis conditions [19,20]. Briefly, analysis was conducted with a Prominence LC-20A series HPLC system (Shimadzu, Kyoto, Japan), which combines a PDA and LCSolution software (Ver. 1.24, SP1; Shimadzu) for data acquisition and processing, respectively. The marker analytes were separated with a SunFire^{TM} C_{18} reverse-phase column (ID 4.6 mm × 250 mm, particle size

5 μm; Waters, Milford, MA, USA) with gradient elution using (A) distilled water and (B) acetonitrile mobile phases, both containing 0.1% (v/v) FA: 5–60% B for 0–40 min, 60–100% B for 40–50 min, 100% B for 50–55 min, and 100–5% B for 55–60 min.

2.6. Validation of Analytical Procedure

Validation of the established analytical method was performed according to the International Conference on Harmonisation guidelines [21]. Namely, the method was assessed based on linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy (recovery), and intra- and interday precisions.

Linearity in the developed assay was confirmed by measuring the coefficient of determination (r^2) based on the calibration curves of each marker component, and LOD and LOQ were calculated by using the equation: LOD = 3.3 × σ/S and LOQ = 10 × σ/S (where σ and S are the standard deviation of the y-intercept and the slope of the calibration curve, respectively).

Recovery tests using the standard addition assay were conducted to verify the accuracy and the results were evaluated using the equation: recovery (%) = found concentration/spiked concentration × 100%.

The precision of the developed HPLC analytical method was determined based on the repeatability, intraday precision, and interday precision and the error was evaluated by calculating the relative standard deviation (RSD) values; the latter were calculated as follows: RSD (%) = standard deviation (SD)/mean × 100%.

3. Results and discussion

3.1. Optimizing Conditions for HPLC Analysis

The optimal chromatographic separation conditions for the simultaneous quantification of eight analytes (MULA, AMY, LIQA, LIQ, NAR, HES, RA, and GA) in HPYJT using HPLC were: SunFire C18 analytical column, column temperature of 40 °C, and distilled water–acetonitrile mobile system (both solvent containing 0.1% (v/v) FA). All analytes eluted under the optimal separation conditions within 40 min with a resolution >4.0. The retention times of the analytes were 12.20, 14.23, 18.75, 19.16, 20.37, 21.55, 23.25, and 36.79 min, respectively. A representative HPLC chromatogram is shown in Figure 1.

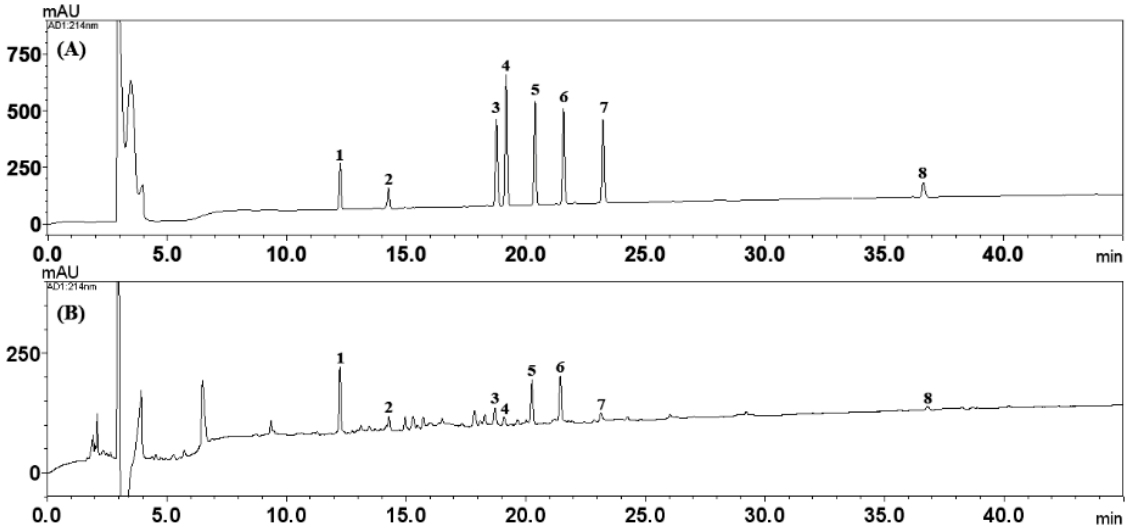

Figure 1. Representative HPLC chromatograms of (**A**) standard solution of all analytes and (**B**) HPYJT sample at 214 nm. (**1**) MULA, (**2**) AMY, (**3**) LIQA, (**4**) LIQ, (**5**) NAR, (**6**) HES, (**7**) RA, and (**8**) GA.

3.2. Validation of the Analytical Procedure

System suitability tests were conducted to ensure the performance of the chromatography system with respect to capacity factor (k'), relative retention (α), theoretical plate number (N), resolution (Rs),

and symmetry factor (*S*). The system and all parameters were found to be suitable for performing the analysis (Table S2). The r^2 values of the eight marker components showed good linearity from 0.9996 to 1.0000, and LOD and LOQ values of these compounds were also calculated to be 0.09–0.48 μg/mL and 0.27–1.46 μg/mL, respectively. These results are summarized in Table 1. Recovery tests, which were conducted to ensure the validity of the method, showed that all the analytes were found with recovery rates from 97% to 103.50% and RSD values of ≤2.50% (Table 2). To evaluate repeatability, the results were analyzed six times, and the RSD values of retention time and peak area showed excellent repeatability of ≤0.11% and ≤0.71%, respectively. Intra- and interday precisions also showed good RSD values below 2.94% (Table 3). The validation parameters reported above confirm that the developed HPLC analytical method is suitable for simultaneous quantification of HPYJT.

Table 1. Linear range, regression equation, r^2, LODs, and LOQs for the eight analytes (n = 3).

Analyte	Linear Range (μg/mL)	Regression Equation [a] $y = ax + b$	r^2	LOD (μg/mL)	LOQ (μg/mL)
MULA	1.56–100.00	$y = 26254.19x + 7269.63$	0.9998	0.35	1.06
AMY	1.56–100.00	$y = 6226.21x + 3363.42$	0.9996	0.09	0.27
LIQA	1.56–100.00	$y = 14121.49x + 5547.89$	0.9999	0.24	0.72
LIQ	0.78–50.00	$y = 18895.76x + 3098.35$	0.9999	0.14	0.42
NAR	1.56–100.00	$y = 16761.20x + 6157.72$	0.9999	0.48	1.46
HES	1.56–100.00	$y = 15716.44x + 6639.20$	0.9999	0.31	0.95
RA	0.78–50.00	$y = 20714.33x + 830.82$	1.0000	0.11	0.33
GA	1.56–100.00	$y = 8011.17x + 2326.31$	0.9999	0.24	0.72

[a] y: peak area (mAU) of each analyte; x: concentration (μg/mL) of each analyte.

Table 2. Recovery test for the assay of the eight marker components in HPYJT (Haepyoyijin-tang).

Analyte	Spiked Conc. (μg/mL)	Found Conc. (μg/mL)	Recovery (%)	SD	RSD (%)
MULA	6.00	5.92	98.68	1.21	1.22
	15.00	15.08	100.52	1.50	1.50
	30.00	31.05	103.50	1.10	1.07
AMY	6.00	5.88	97.99	2.49	2.54
	15.00	14.99	99.94	2.35	2.36
	30.00	30.18	100.61	1.81	1.80
LIQA	4.00	4.02	100.54	0.95	0.95
	10.00	9.81	98.11	1.54	1.56
	20.00	20.45	102.27	0.71	0.69
LIQ	2.00	1.97	98.39	2.14	2.17
	5.00	4.90	97.98	1.01	1.03
	10.00	10.00	100.05	0.62	0.62
NAR	4.00	3.97	99.25	2.57	2.59
	10.00	9.72	97.18	1.08	1.11
	20.00	19.91	99.56	1.16	1.16
HES	4.00	3.98	99.54	2.52	2.53
	10.00	10.09	100.94	1.94	1.92
	20.00	19.84	99.22	1.33	1.34
RA	2.00	2.01	100.30	1.38	1.37
	5.00	4.93	98.66	1.12	1.13
	10.00	10.21	102.05	1.00	0.98
GA	4.00	4.01	100.26	0.54	0.54
	10.00	9.80	98.01	1.62	1.65
	20.00	20.65	103.26	1.11	1.07

Table 3. Precision assay for the eight analytes in HPYJT.

Analyte	Conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)			Repeatability (n = 6)	
		Measured Conc. (μg/mL)	Precision (%)	Accuracy (%)	Measured Conc. (μg/mL)	Precision (%)	Accuracy (%)	RSD (%) of Retention Time	RSD (%) of Peak Area
MULA	25.00	25.25	0.30	101.02	25.17	0.94	100.69	0.11	0.51
	50.00	50.79	0.75	101.57	51.72	2.94	103.44		
	100.00	101.10	0.64	101.10	102.38	1.18	102.38		
AMY	25.00	24.93	0.98	99.71	24.88	1.01	99.50	0.04	0.71
	50.00	50.22	0.39	100.43	50.60	1.24	101.20		
	100.00	100.53	1.42	100.53	100.55	1.32	100.55		
LIQA	25.00	25.46	0.39	101.84	25.36	0.77	101.46	0.02	0.46
	50.00	50.87	0.62	101.74	51.80	2.41	103.60		
	100.00	101.23	0.75	101.23	102.30	1.14	102.30		
LIQ	12.50	12.69	0.83	101.53	12.61	0.83	100.92	0.02	0.50
	25.00	25.35	0.93	101.41	25.88	2.83	103.52		
	50.00	50.34	0.60	100.69	50.82	1.01	101.63		
NAR	25.00	25.39	0.49	101.57	25.30	0.69	101.20	0.04	0.45
	50.00	50.69	0.39	101.38	51.11	1.09	102.22		
	100.00	101.00	0.28	101.00	101.93	0.91	101.93		
HES	25.00	25.44	0.35	101.76	25.35	0.66	101.39	0.05	0.47
	50.00	50.85	0.42	101.70	51.16	0.97	102.31		
	100.00	101.06	0.58	101.06	102.08	1.03	102.08		
RA	12.50	12.38	0.34	99.02	12.24	1.07	97.94	0.05	0.47
	25.00	24.88	0.75	99.51	25.10	1.85	100.38		
	50.00	49.98	0.62	99.96	50.09	0.58	100.18		
GA	25.00	25.24	0.17	100.98	25.12	0.69	100.48	0.01	0.47
	50.00	50.41	0.36	100.83	51.25	2.55	102.51		
	100.00	100.38	0.55	100.38	100.46	0.71	100.46		

3.3. Simultaneous Determination of the Eight Marker Analytes in HPYJT

The developed and validated HPLC analytical method was applied for the quantitative analysis of the eight analytes in the lyophilized HPYJT sample. Quantification was performed simultaneously by scanning a wavelength range of 190–400 nm with a PDA detector. The wavelength monitored for the quantitative analysis of each component was: 214 nm for AMY, 254 nm for GA, 275 nm for LIQA and LIQ, 280 nm for NAR and HES, 325 nm for MULA, and 330 nm for RA. In the lyophilized HPYJT sample, the eight marker compounds were found to be present in 0.43 mg/g to 2.83 mg/g and, among these, it was confirmed that MULA was the most abundant in the HPYJT sample at 2.83 mg/g (Table 4).

Table 4. Quantification of the eight marker components in HPYJT sample.

Compound	Content (mg/g)	
	Mean ± SD	RSD (%)
MULA	2.83 ± 0.06	2.06
AMY	2.77 ± 0.02	0.71
LIQA	1.49 ± 0.02	1.68
LIQ	0.44 ± 0.01	2.35
NAR	2.20 ± 0.03	1.49
HES	2.54 ± 0.06	2.33
RA	0.43 ± 0.01	2.26
GA	2.39 ± 0.04	1.61

4. Conclusions

Simultaneous analysis of a HPYJT sample was performed using HPLC–PDA to establish an efficient quality control protocol. The analytical methods were developed and verified with respect to parameters such as linearity, LOD, LOQ, accuracy (recovery), and precision. It is anticipated that the established analytical method will be used to obtain basic data for the quality assessment of HPYJT and related herbal formulas.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/11/3888/s1, Figure S1: Chemical structures of the eight marker components in HPYJT; Table S1: Composition of HPYJT; Table S2: System suitability of eight analytes for HPLC analysis.

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 a grant from the Korea Institute of Oriental Medicine (grant number KSN1812230).

Conflicts of Interest: The authors have declared no conflicts of interest.

References

1. Heo, J. *Dongui Bogam*; Namsandang: Seoul, Korea, 2007; p. 481.
2. Lee, K.T.; Park, D.I. The effects of Haepyoy jintang on the pulmonary injury caused by SO_2 in rats. *J. Korean Med.* **1996**, *17*, 178–190.
3. Suk, Y.H.; Min, S.Y.; Kim, J.H. Effect of Haepyo Jin-tang on Airway Mucin Secretion, Production, Gene Expression and Hypersecretion of Mucus. *J. Korean Pediatr. Med.* **2015**, *29*, 65–79. [CrossRef]
4. Han, J.H.; Jo, S.G.; Lee, M.J.; Baek, S.H.; Park, S.H. Contents of homogentisic acid and 3,4-dihydroxybenzaldehyde in the Pinellia ternate by various processing method and its safety estimate. *J. Physiol. Pathol. Korean Med.* **2004**, *18*, 846–853.
5. Zhao, B.T.; Kim, E.J.; Son, K.H.; Son, J.K.; Min, B.S.; Woo, M.H. Quality evaluation and pattern recognition analyses of marker compounds from five medicinal drugs of Rutaceae family by HPLC/PDA. *Arch. Pharm. Res.* **2015**, *38*, 1512–1520. [CrossRef] [PubMed]

6. Li, G.; Xu, M.-L.; Lee, C.-S.; Woo, M.-H.; Chang, H.-W.; Son, J.K. Cytotoxicity and DNA topoisomerases inhibitory activity of constituents from the sclerotium of Poria cocos. *Arch. Pharm. Res.* **2004**, *27*, 829–833. [CrossRef] [PubMed]
7. Zhang, Q.; Ye, M. Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). *J. Chromatogr.* **2009**, *1216*, 1954–1969. [CrossRef] [PubMed]
8. Zhao, B.T.; Lee, K.R.; Lee, J.H.; Min, B.S.; Son, J.K.; Woo, M.H. Quality evaluation of Perillae Folium by HPLC/PDA. *Arch. Pharm. Res.* **2015**, *38*, 1521–1529. [CrossRef] [PubMed]
9. Okamura, N.; Miki, H.; Harada, T.; Yamashita, S.; Masaoka, Y.; Nakamoto, Y.; Tsuguma, M.; Yoshitomi, H.; Yagi, A. Simultaneous determination of ephedrine, pseudoephedrine, norephedrine and methylephedrine in Kampo medicines by high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* **1999**, *20*, 363–372. [CrossRef]
10. Koo, J.-Y.; Hwang, E.-Y.; Cho, S.; Lee, J.-H.; Lee, Y.-M.; Hong, S.-P. Quantitative determination of amygdalin epimers from armeniacae semen by liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2005**, *814*, 69–73. [CrossRef] [PubMed]
11. Kim, K.T.; Shin, M.C.; Cho, C.W.; Lee, W.J.; Woo, E.R.; Kang, J.S. Specification and Analysis of Multiple Marker Compounds for Quality Control of Mori Cortex Radicis by HPLC. *Bull. Korean Chem. Soc.* **2015**, *36*, 117–122. [CrossRef]
12. Chung, K.-O.; Kim, B.-Y.; Lee, M.-H.; Kim, Y.-R.; Chung, H.-Y.; Park, J.-H.; Moon, J.-O. In-vitro and in-vivo anti-inflammatory effect of oxyresveratrol from Morus alba L. *J. Pharm. Pharmacol.* **2003**, *55*, 1695–1700. [CrossRef] [PubMed]
13. Yang, H.; Shi, H.; Zhang, Q.; Liu, Y.; Wan, C.; Zhang, L. Simultaneous Determination of Five Components in Aster tataricus by Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. Sci.* **2015**, *54*, 500–506. [CrossRef] [PubMed]
14. Zhao, D.X.; Hu, B.Q.; Zhang, M.; Zhang, C.F.; Xu, X.H. Simultaneous separation and determination of phenolic acids, pentapeptides, and triterpenoid saponins in the root of Aster tataricus by high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry. *J. Sep. Sci.* **2015**, *38*, 571–575. [CrossRef] [PubMed]
15. Chinese Pharmacopoeia Commission. *Pharmacopoeia of the People's Republic of China*; China Medical Science Press: Beijing, China, 2015; p. 292.
16. Ha, Y.W.; Kim, Y.S. Preparative isolation of six major saponins from Platycodi Radix by high-speed counter-current chromatography. *Phytochem. Anal.* **2009**, *20*, 207–213. [CrossRef] [PubMed]
17. Wang, W.; Li, C.-Y.; Wen, X.; Qi, L.-W.; Qi, L.-W. Simultaneous determination of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol in rat plasma by liquid chromatography–mass spectrometry: Application to pharmacokinetics. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2009**, *877*, 671–679. [CrossRef] [PubMed]
18. Lee, K.H. *The Dispensatory on the Visual and Organoleptic Examination of Herbal Medicine*; National Institute of Food and Drug Safety Evaluation: Seoul, Korea, 2013; pp. 24–696.
19. Seo, C.-S.; Shin, H.-K. Quality assessment of traditional herbal formula, Hyeonggaeyeongyo-tang through simultaneous determination of twenty marker components by HPLC-PDA and LC-MS/MS. *Saudi Pharm. J.* **2020**, *28*, 427–439. [CrossRef] [PubMed]
20. Seo, C.-S.; Lee, M.-Y. HPLC–PDA and LC–MS/MS Analysis for the Simultaneous Quantification of the 14 Marker Components in Sojadodamgangki-Tang. *Appl. Sci.* **2020**, *10*, 2804. [CrossRef]
21. International Conference on Harmonisation. *Guidance for Industry, Q2B, Validation of Analytical Procedures: Methodology*; Food and Drug Administration: Rockville, MD, USA, 1996.