Simultaneous Analysis for Quality Control of Traditional Herbal Medicine, Gungha-Tang, Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Gungha-tang (GHT), a traditional herbal medicine, consists of nine medicinal herbs (Cnidii Rhizoma, Pinelliae Tuber, Poria Sclerotium, Citri Unshiu Pericarpium, Citri Unshiu Pericarpium Immaturus, Aurantii Fructus Immaturus, Atracylodis Rhizoma Alba, Glycyrrhizae Radix et Rhizoma, and Zingiberis Rhizoma Recens). It has been used for various diseases caused by phlegm. This study aimed to develop and verify the simultaneous liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis method, using nine marker components (liquiritin apioside, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, liquiritigenin, glycyrrhizin, and 6-shogaol) for quality control of GHT. LC–MS/MS analysis was conducted using a Waters TQ-XS system. All marker analytes were separated on a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) using gradient elution with a distilled water solution (containing 5 mM ammonium formate and 0.1% [v/v] formic acid)–acetonitrile mobile phase. LC–MS/MS multiple reaction monitoring (MRM) analysis was carried out in negative and positive ion modes of an electrospray ionization source. The developed LC–MS/MS MRM method was validated by examining the linearity, limits of detection and quantification, recovery, and precision. LOD and LOQ values of nine markers were calculated as 0.02–8.33 ng/mL and 0.05–25.00 ng/mL. The recovery was determined to be 89.00–118.08% and precision was assessed with a coefficient of variation value of 1.74–8.64%. In the established LC–MS/MS MRM method, all markers in GHT samples were detected at 0.003–16.157 mg/g. Information gathered during the development and verification of the LC–MS/MS method will be useful for the quality assessment of GHT and other herbal medicines.

Keywords: simultaneous analysis; quality control; Gungha-tang; LC–MS/MS

1. Introduction

Traditional herbal medicines, traditional Korean medicines (TKMs), traditional Chinese medicines (TCMs), and Kampo medicines (KMs), characterized by multiple components and multiple targets, have long been used in Asian countries, especially Korea, China, and Japan, for the treatment of and protection against various diseases, and for maintaining health [1]. These TKMs, TCMs, and KMs consist of combinations of at least two or more medicinal herbs and are taken in the form of decoction [2,3].

Gungha-tang (GHT), one of these traditional herbal medicines, consists of nine medicinal herbs: Cnidii Rhizoma, Pinelliae Tuber, Poria Sclerotium, Citri Unshiu Pericarpium, Citri Unshiu Pericarpium Immaturus, Aurantii Fructus Immaturus, Atracylodis Rhizoma Alba, Glycyrrhizae Radix et Rhizoma, and Zingiberis Rhizoma Recens. GHT was first recorded in Ren Zhai Zhi Zhi Fang Lun written by Shiying Yang, a medical doctor from Southern Song Dynasty [4], and thereafter in Dong Eui Bo Gam written by Jun Heo during the Joseon Dynasty. GHT is reported to be used for diseases caused by phlegm [5].

Nine herbal medicines have been reported to have various biological effects, for example, anti-inflammatory, antioxidant, anticancer, antitumor, antidiabetes, antiaging,
anti-obesity, neuroprotective, and antibacterial activities [6–14]. Recently, a study on the safety of a single administration of GHT was reported by An et al. [4], but few studies on the biological activity have been reported.

The main ingredients of GHT, composed of nine herbs, are the following: chlorogenic acid, ferulic acid, senkyunolide A, and Z-ligustilide from Cnidii Rhizoma; homogentisic acid and 3,4-dihydroxybenzaldehyde from Pinelliae Tuber; pachymic acid, dehydrotumulosic acid, and polypropenic acid C from Poria Sclerotium; naringin, hesperidin, and narirutin from Citri Unshiu Pericarpium, Citri Unshiu Pericarpium Immaturus, and Aurantii Fructus Immaturus; atracylenolide I and III from Atractylodis Rhizoma Alba; liquiritin, liquiritin apioside, and glycyrrhizin from Glycyrrhizae Radix et Rhizoma; and 6-gingerol from Zingiberis Rhizoma Recens [15–23]. Analytical studies to determine qualitative, quantitative, or chemical profiling analyses have been conducted to evaluate the quality of each herb using high-performance liquid chromatography (HPLC) or liquid chromatography–tandem mass spectrometry (LC–MS/MS) systems for the main active components of each herbal medicine mentioned above [15–23]. However, no studies have been reported for the quality control of GHT composed of a combination of these nine herbal medicines.

To date, many researchers have used analytical techniques such as HPLC, LC–MS/MS, and gas chromatography–mass spectrometry for the quality control of complex formulations such as TKMs, TCMs, and KMs [24–28]. Among the various analytical techniques, the analytical methods that include HPLC and LC–MS/MS are currently the most widely used for standardization purposes. In particular, the sensitive, accurate, and reliable LC–MS/MS system is being used in standardization studies for numerous phytochemical components that constitute TKMs, TCMs, and KMs [29].

Therefore, in the present study, a simultaneous analysis method was developed, and then verified, utilizing the LC–MS/MS multiple reaction monitoring (MRM) assay, for efficient quality control of GHT using the following nine marker components: liquiritin apioside, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, liquiritigenin, glycyrrhizin, and 6-shogaol.

2. Results and Discussion

2.1. Optimization of LC–MS/MS MRM Conditions

For the quality assessment of GHT using the marker analytes, we first determined the optimal simultaneous determination conditions in LC–MS/MS MRM mode. Consequently, the nine markers (liquiritin apioside, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, liquiritigenin, glycyrrhizin, and 6-shogaol) were separated using gradient elution with a distilled water solution (containing 5 mM ammonium formate and 0.1% \(v/v\) formic acid)–acetonitrile mobile phase system on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) maintained at 45 °C. Table 1 shows the optimal LC–MS/MS MRM parameters for the simultaneous quantification of each marker component. The established assay was successfully applied to the GHT sample and all markers were detected within 10 min, as shown in Figure 1 and Table 1.

| Compound         | Ion Mode | Molecular Weight | Precursor Ion (Q1) | Product Ion (Q3) | Cone Voltage (V) | Collision Energy (eV) | Retention Time (min) |
|------------------|----------|------------------|-------------------|-----------------|-----------------|-----------------------|---------------------|
| Liqiritin apioside | −        | 550.2            | 549.3             | 255.0           | 45              | 30                    | 1.57                |
| Neoeriocitrin     | ‒        | 596.2            | 595.5             | 151.0           | 30              | 40                    | 1.58                |
| Narirutin         | +        | 580.2            | 581.0             | 273.0           | 15              | 15                    | 1.86                |
| Naringin          | −        | 580.2            | 579.3             | 271.0           | 45              | 30                    | 1.99                |
| Hesperidin        | +        | 610.2            | 611.5             | 303.2           | 20              | 15                    | 2.13                |
| Neohesperidin     | +        | 610.2            | 611.0             | 303.0           | 15              | 20                    | 2.27                |
| Liquiritigenin    | +        | 256.1            | 257.2             | 137.0           | 35              | 35                    | 3.05                |
| Glycyrrhizin      | −        | 822.4            | 821.9             | 351.2           | 45              | 40                    | 4.95                |
| 6-Shogaol         | +        | 276.2            | 277.2             | 137.1           | 25              | 15                    | 8.50                |
Figure 1. Total ion chromatograms of mixed standard solution of the nine marker components (A) and 70% methanolic solution of the freeze-dried GHT water decoction sample (B) acquired by LC–MS/MS MRM in positive and negative ion modes. Liquiritin apioside (1), neoeriocitrin (2), narirutin (3), naringin (4), hesperidin (5), neohesperidin (6), liquiritigenin (7), glycyrrhizin (8), and 6-shogaol (9).

2.2. Identification of Each Marker Analyte for LC–MS/MS MRM Analysis

In the LC–MS/MS MRM analysis of each marker analyte, use was made of an electro-spray ionization source of negative and positive ion modes. Four components (liquiritin apioside, neoeriocitrin, naringin, and glycyrrhizin) were detected at m/z 549.3, 595.5, 579.3, and 821.9, respectively, in the form of [M–H]−, negative ion mode, and the remaining five components (narirutin, hesperidin, neohesperidin, liquiritigenin, and 6-shogaol) were detected at m/z 581.0, 611.5, 611.0, 257.2, and 277.2, respectively, in the form of [M+H]+, positive ion mode (Figure 1, Table 1). As MRM conditions for LC–MS/MS simultaneous analysis, the precursor ion (Q1) and product ion (Q3) peaks for each marker analyte were set as shown in Table 1. Liquiritin apioside, a flavanone, detected a Q3 ion peak at m/z 255.0 (M–H–Glc-Api)−, generated by removal of the glucosyl-apiosyl group [30]. Q3 ion peaks of narirutin, naringin, hesperidin, and neohesperidin were detected at m/z 273.0 ([M+H–Glc-Rham]−), 271.0 ([M–H–Glc-Rham]−), 303.2 ([M+H–Glc-Rham]+), and 303.0 ([M+H–Glc-Rham]+), respectively, in the form of an aglycone from which rutinose was eliminated [31–33]. Neoeriocitrin was detected at m/z 151.0 in the form of [1,3-A0–H]−, generated by retro-Diels–Alder (RDA) fragmentation of aglycone, from which rutinose had been removed [31,32]. The fragmentation of liquiritigenin is similar to that of neoeriocitrin; the Q3 ion peak was detected at m/z 137.0 ([M+H–4-vinylphenol]+) by RDA cleavage [31]. Glycyrrhizin was in the form of di-GlcA–H−, in which aglycone was lost, and a Q3 ion peak was detected at m/z 351.2 [30]. In 6-shogaol, the Q3 ion peak was detected at m/z 137.1 in the form of [M+H–C9H15O]+, by cleavage of the C1–C2 bond by the ketone functional group of the alkyl chain [34,35]. MS fragmentation for the simultaneous determination of each marker as described above is shown in Figure S2.
2.3. Method Validation of the Developed Analytical Method

In this research, the newly developed LC–MS/MS MRM analytical method for the simultaneous determination of the nine marker analytes in GHT samples was validated by evaluating the linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision. Table 2, Table 3, Table 4 show the results for various validation parameters. Briefly, the coefficient of determination ($r^2$) value, which means the linearity of the calibration curve prepared in different concentration ranges for each marker analyte, was 0.9950–0.9968, showing good linearity, and LOD and LOQ values were estimated to be 0.02–8.33 ng/mL and 0.05–25.00 ng/mL, respectively (Table 2). The recovery test, calculated from Equation (1), was conducted to evaluate the accuracy of the developed method; it was determined to be 89.00–118.08% (Table 3). The acceptance criteria for recovery test for validation of analysis of traditional herbal medicines such as TKMs, TCMs, and KMs are generally accepted with ±20%, so results of our study show that they are suitable [36,37]. In the precision verification, the repeatability of the retention time and peak area of the marker analytes was evaluated as the coefficient of variation (CV) values (calculated from Equation (2)); it was determined to be 0.08–0.52% and 3.04–9.64%, respectively (Table S2). In addition, the CV (%) values of intra- and interday precisions of the nine marker analytes were also determined to be <10.00% (Table 4). From the above various verification results, indications are that the LC–MS/MS MRM assay developed in this study is suitable and appropriate as an analytical method for the quality evaluation of GHT.

Table 2. Various parameters for simultaneous determination of marker analytes in GHT using the LC–MS/MS MRM assay.

| Analyte               | Linear Range (ng/mL) | Regression Equation $y=ax+b$ | $r^2$ | LOD (ng/mL) | LOQ (ng/mL) |
|-----------------------|-----------------------|-------------------------------|-------|-------------|-------------|
| Liquiritin apioside   | 25.00–400.00          | $y = 68.42x + 52.85$         | 0.9968| 8.33        | 25.00       |
| Neoeriocitrin         | 50.00–800.00          | $y = 48.69x - 80.24$         | 0.9958| 0.83        | 2.50        |
| Narirutin             | 50.00–800.00          | $y = 20.32x + 202.74$        | 0.9954| 3.33        | 10.00       |
| Naringin              | 50.00–800.00          | $y = 23.58x - 107.24$        | 0.9950| 8.33        | 25.00       |
| Hesperidin            | 50.00–800.00          | $y = 153.88x + 509.37$       | 0.9951| 1.67        | 5.00        |
| Neohesperidin         | 100.00–1600.00        | $y = 29.03x + 905.96$        | 0.9950| 0.33        | 1.00        |
| Liquiritigenin        | 0.10–1.60             | $y = 19,647.00x + 239.15$   | 0.9959| 0.02        | 0.05        |
| Glycyrrhizin          | 50.00–800.00          | $y = 14.51x - 40.59$         | 0.9953| 1.67        | 5.00        |
| 6-Shogaol             | 0.10–1.60             | $y = 19,566.10x + 659.29$   | 0.9966| 0.02        | 0.05        |

*a: $y$: peak area of each analyte; $x$: concentration of each analyte.

Table 3. Recovery tests for each marker analyte in GHT using the developed LC–MS/MS MRM assay.

| Analyte       | Spiked Amount (ng/mL) | Amount Found (ng/mL) | Recovery (%) | SD  | CV (%) |
|---------------|-----------------------|----------------------|--------------|-----|--------|
| Liquiritin apioside | 200.00               | 234.34               | 117.17       | 1.44| 1.23   |
|               | 400.00               | 458.24               | 114.56       | 3.30| 2.88   |
|               | 800.00               | 862.88               | 107.86       | 0.78| 0.72   |
|               | 50.00                | 59.04                | 118.08       | 1.31| 1.11   |
| Neoeriocitrin | 100.00               | 110.50               | 110.50       | 4.10| 3.71   |
|               | 200.00               | 219.08               | 109.54       | 3.74| 3.41   |
| Narirutin     | 500.00               | 557.52               | 111.50       | 2.36| 2.02   |
|               | 1000.00              | 1050.68              | 105.07       | 3.17| 1.30   |
| Naringin      | 500.00               | 573.90               | 114.78       | 2.84| 2.47   |
|               | 1000.00              | 1157.74              | 115.77       | 2.10| 1.81   |
|               | 2000.00              | 2237.84              | 111.89       | 1.97| 1.76   |
Table 3. Cont.

| Analyte          | Spiked Amount (ng/mL) | Amount Found (ng/mL) | Recovery (%) | SD  | CV (%) |
|------------------|-----------------------|----------------------|--------------|-----|--------|
| Hesperidin       | 500.00                | 544.38               | 108.88       | 1.75| 1.61   |
|                  | 1000.00               | 1013.86              | 101.39       | 2.72| 2.68   |
|                  | 2000.00               | 1983.78              | 99.19        | 1.72| 1.73   |
|                  | 4000.00               | 4121.26              | 103.03       | 3.02| 2.94   |
| Neohesperidin    | 2000.00               | 2009.60              | 100.48       | 5.05| 5.03   |
|                  | 4000.00               | 4012.36              | 100.34       | 5.04| 5.03   |
| Liquiritigenin   | 8.00                  | 7.88                 | 98.50        | 6.75| 6.86   |
|                  | 16.00                 | 14.50                | 96.25        | 7.51| 7.62   |
| Glycyrrhizin     | 1000.00               | 1173.10              | 117.31       | 2.13| 2.14   |
|                  | 2000.00               | 2174.32              | 108.72       | 1.38| 1.39   |
| 6-Shogaol        | 2.00                  | 1.90                 | 95.00        | 7.91| 8.02   |
|                  | 4.00                  | 3.56                 | 89.00        | 7.62| 7.73   |

Table 4. Precision data for simultaneous determination of the nine marker analytes in the developed LC–MS/MS MRM assay.

| Analyte          | Conc. (ng/mL) | Intraday (n = 5) | Interday (n = 5) |
|------------------|---------------|------------------|------------------|
|                  | Obtained Conc. (ng/mL) | Precision (%) | Accuracy (%) | Obtained Conc. (ng/mL) | Precision (%) | Accuracy (%) |
| Liquiritin       | 200.00        | 180.82           | 3.58            | 90.41          | 201.70      | 2.32          | 100.85       |
| apiside          | 400.00        | 385.94           | 3.64            | 96.49          | 407.92      | 3.21          | 101.98       |
|                  | 800.00        | 784.44           | 4.66            | 98.06          | 807.92      | 2.45          | 100.99       |
|                  | 50.00         | 44.56            | 5.35            | 89.12          | 50.08       | 3.34          | 100.15       |
| Neoeiricitrin    | 100.00        | 96.76            | 5.10            | 96.76          | 99.12       | 4.96          | 99.12        |
|                  | 200.00        | 204.04           | 2.10            | 102.02         | 203.84      | 2.62          | 101.92       |
|                  | 500.00        | 464.20           | 6.88            | 92.84          | 502.50      | 3.86          | 100.50       |
| Narirutin        | 1000.00       | 940.26           | 4.62            | 94.03          | 976.10      | 3.51          | 97.61        |
|                  | 2000.00       | 1673.18          | 8.45            | 83.66          | 1868.60     | 3.92          | 93.43        |
|                  | 500.00        | 470.92           | 2.57            | 94.18          | 500.80      | 2.23          | 100.16       |
| Naringin         | 1000.00       | 972.82           | 2.11            | 97.28          | 1008.50     | 1.89          | 100.85       |
|                  | 2000.00       | 2020.58          | 1.74            | 101.03         | 2019.00     | 1.85          | 100.95       |
|                  | 500.00        | 465.32           | 2.39            | 93.06          | 492.55      | 2.43          | 98.51        |
| Hesperidin       | 1000.00       | 939.70           | 3.16            | 93.97          | 967.20      | 2.74          | 96.72        |
|                  | 2000.00       | 1982.78          | 3.06            | 99.14          | 1967.20     | 1.85          | 98.36        |
|                  | 1000.00       | 955.86           | 5.67            | 95.59          | 987.40      | 5.54          | 98.74        |
| Neohesperidin    | 2000.00       | 1948.32          | 5.38            | 97.42          | 1929.40     | 5.04          | 96.47        |
|                  | 4000.00       | 3908.70          | 3.86            | 97.72          | 3997.20     | 3.05          | 99.93        |
|                  | 4.00          | 3.74             | 4.06            | 93.50          | 3.55        | 5.76          | 88.83        |
| Liquiritigenin   | 8.00          | 7.30             | 3.21            | 91.25          | 7.77        | 4.85          | 97.08        |
|                  | 16.00         | 14.98            | 5.41            | 93.63          | 15.27       | 5.87          | 95.46        |
|                  | 500.00        | 511.30           | 5.38            | 102.26         | 517.31      | 3.36          | 103.46       |
| Glycyrrhizin     | 1000.00       | 971.88           | 3.86            | 97.19          | 1027.81     | 2.62          | 102.78       |
|                  | 2000.00       | 1976.92          | 3.40            | 98.85          | 2031.49     | 2.34          | 101.57       |
|                  | 1.00          | 0.98             | 8.54            | 98.00          | 0.92        | 8.64          | 92.00        |
| 6-Shogaol        | 2.00          | 1.74             | 6.55            | 87.00          | 1.87        | 7.72          | 93.67        |
|                  | 4.00          | 3.88             | 8.43            | 97.00          | 4.05        | 6.17          | 101.17       |

* Precision (%) is expressed as CV (%) calculated from Equation (2).
2.4. Simultaneous Determination of the Nine Marker Analytes in GHT Samples Using the Developed LC–MS/MS MRM Assay

Simultaneous determination of the nine marker analytes in GHT samples was conducted using the LC–MS/MS MRM assay developed and validated in this study. All marker analytes (liquiritin apioside, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, liquiritigenin, glycyrrhizin, and 6-shogaol) were eluted, at 1.57, 1.58, 1.86, 1.99, 2.13, 2.27, 3.05, 4.95, and 8.50 min, respectively (Table 1 and Figure S3). The nine marker analytes in the lyophilized GHT samples were detected at 0.003–16.157 mg/g. The detailed content of each marker compound is given in Table 5. Among these markers, narirutin, naringin, hesperidin, and neohesperidin, derived from Citri Unshiu Pericarpium, Citri Unshiu Pericarpium Immaturus, Aurantii Fructus Immaturus, were present in large amounts. These results suggest the possibility of them being useful as basic data for the analysis of quality assessment of GHT.

Table 5. Amounts of the nine marker analytes in GHT samples by LC–MS/MS MRM assay (n = 3).

| Analyte               | GHT-1 a |          |          |          | GHT-2 b |          |          |
|-----------------------|---------|----------|----------|----------|---------|----------|----------|
|                       | Mean    | SD       | CV (%)   | Mean     | SD       | CV (%)   |
| Liquiritin apioside   | 0.007   | 0.002    | 2.831    | 0.003    | 0.002    | 8.377    |
| Neoeriocitrin         | 1.089   | 0.259    | 2.377    | 0.315    | 0.292    | 9.274    |
| Narirutin             | 5.878   | 2.395    | 4.075    | 0.944    | 0.838    | 8.870    |
| Naringin              | 16.157  | 1.297    | 0.803    | 2.785    | 2.473    | 8.880    |
| Hesperidin            | 8.002   | 1.647    | 2.058    | 6.559    | 1.031    | 1.573    |
| Neohesperidin         | 8.338   | 5.982    | 7.175    | 0.044    | 0.035    | 7.857    |
| Liquiritigenin        | 2.423   | 0.518    | 2.139    | 0.809    | 0.741    | 9.160    |
| Glycyrrhizin          | 6.416   | 0.818    | 1.275    | 2.801    | 0.819    | 2.923    |
| 6-Shogaol             | 0.007   | 0.007    | 9.042    | 0.008    | 0.007    | 8.072    |

a GHT-1: sample was prepared in Korea Institute of Oriental Medicine; b GHT-2: sample was made by commercial pharmaceutical company.

3. Materials and Methods
3.1. Plant Materials

Nine raw herbal medicines constituting GHT were purchased from Kwangmyungdang Medicinal Herbs (KMH; Ulsan, Korea), a herbal medicine supplier for pharmaceuticals, in November 2017. All medicinal herbs were used after morphological verification by Dr. Seung-Yeol Oh, president of KMH. Detailed information on all raw herbs is shown in Table S1. Specimens of the nine raw herbal medicines (2017KE58–1 to 2017KE58–5) were deposited at the KM Science Research Division, Korea Institute of Oriental Medicine.

3.2. Chemicals and Reagents

The nine standard compounds used as markers for quality assessment of GHT in this study are shown in Figure S1. These compounds were provided by commercial suppliers and used for LC–MS/MS analysis: liquiritin apioside (C_{26}H_{30}O_{13}, CAT No. DR10690, 99.6%), hesperidin (C_{28}H_{34}O_{15}, CAT No. DR10882, 98.7%), neohesperidin (C_{28}H_{34}O_{15}, CAT No. DR10883, 98.4%), and 6-shogaol (C_{17}H_{24}O_{3}, CAT No. DR10924, 99.2%) from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China); neoeriocitrin (C_{27}H_{32}O_{15}, CAT No. TBW00746, 99.9%) from Wuhan ChemNorm Biotech Co., Ltd. (Wuhan, China); narirutin (C_{27}H_{32}O_{14}, CAT No. BP0985, 99.5%), liquiritigenin (C_{15}H_{12}O_{4}, CAT No. BP0873, 99.8%), and glycyrrhizin (C_{42}H_{62}O_{16}, CAT No. BP0682, 99.1%) from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China); naringin (C_{27}H_{32}O_{14}, CAT No. 71162, 95.0%) from KGaA (Darmstadt, Germany). Methanol and acetonitrile were LC–MS grade and supplied by ThermoFisher Scientific (San Jose, CA, USA). Purified water was used, specifically, produced through a Vivagen water purification system (EXL3 Analysis 16, Seongnam, Korea).
Formic acid (≥99.5%) was supplied by Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan) and ammonium formate (99.0%) by Kanto Chemical Co., Inc. (Tokyo, Japan).

3.3. Preparation of GHT Water Sample

A sample of GHT in water was prepared following the same protocol as that in other previously reported methods for preparing a herbal prescription [38] (see Table S1). After mixing nine herbal medicines (Cnidii Rhizoma, Pinelliae Tuber, and Poria Sclerotium, each 881.52 g; Citri Unshiu Pericarpium, Citri Unshiu Pericarpium Immaturus, and Aurantii Fructus Immaturus, each 441.94 g; Atractylodis Rhizoma Alba and Glycyrrhizae Radix et Rhizoma, each 220.97 g; and Zingiberis Rhizoma Recens, 587.68 g), in a weight ratio (w/w), 50 L of distilled water was added, and extraction was performed under pressure (0.98 bar) at 100 °C for 2 h. The extract was subsequently filtered through a sieve (53 µm mesh) and then freeze dried (PVTFD100R, IlshinBioBase, Yangju, Korea) to afford a powder sample of about 1.0 kg (yield 20.1%).

3.4. Preparation of Samples and Standard Solutions for LC–MS/MS Quantification of the Nine Marker Analytes in GHT Samples

To analyze simultaneously the nine marker analytes in a GHT sample using LC–MS/MS, 70% methanol was added to approximately 50 mg of the lyophilized GHT sample to make up a volume of 50 mL. The mixed sample solution was continuously subjected to ultrasonic extraction for 5 min and vortexing for 1 min. Prior to analysis, the prepared sample solution was diluted 50-fold with 70% methanol and filtered through a hydrophobic polytetrafluoroethylene membrane filter (0.2 µm; Pall Life Sciences, Ann Arbor, MI, USA).

A standard solution for each marker analyte was prepared at a concentration of 100.0 µg/mL, using methanol, and then stored at 4 °C until analysis.

3.5. LC–MS/MS Instrumentation and Experimental Conditions for Simultaneous Determination of the Nine Marker Analytes in GHT Samples

Simultaneous determination of the nine marker analytes in GHT samples by LC–MS/MS was conducted using a previously reported protocol [39,40]. Briefly, LC–MS/MS analysis was performed using a Waters Acquity UPLC system (Milford, MA, USA) coupled with a Waters Xevo TQ-XS triple quadrupole MS system. Markers were separated on an Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm), maintained at 45 °C, using gradient elution with a distilled water solution (containing 5 mM ammonium formate and 0.1% [v/v] formic acid)–acetonitrile mobile phase system. Detailed experimental parameters of ultra-performance liquid chromatography and MS for simultaneous determination are summarized in Table S3.

3.6. Method Validation of the Developed LC–MS/MS MRM Assay

The simultaneous LC–MS/MS analysis method developed for the consistent quality control of GHT was verified by evaluating linearity, LOD, LOQ, accuracy (recovery), and precision (repeatability, intraday precision, and interday precision). Verification of linearity was evaluated by the $r^2$ of the calibration curve prepared at different concentrations of each marker analyte: 25.00–400.00 ng/mL (liquiritin apioside), 50.00–800.00 ng/mL (neoeriocitrin, narirutin, naringin, hesperidin, and glycyrrhizin), 100.00–1600.00 ng/mL (neohesperidin), and 0.10–1.60 ng/mL (liquiritigenin and 6-shogaol). LOD and LOQ were automatically calculated by the LC–MS/MS system (MassLynx software, version 4.2, Waters, Milford, MA, USA) as a signal-to-noise ratios of 3 and 10, respectively.

The accuracy verification of the newly developed LC–MS/MS method was performed through the recovery test. In other words, the recovery (%) was determined by adding known concentrations of each standard marker analyte (low, medium, and high) to the GHT sample as shown in Table 3, and calculated from Equation (1).
Recovery (%) = \frac{\text{amount found}}{\text{spiked amount}} \times 100 \quad (1)

The precision (repeatability, intraday precision, and interday precision) of our newly developed analytical method was evaluated by calculating the CV value of each parameter. The repeatability was validated by calculating the CV value of retention time and peak area of each marker analyte, after six measurements, using a standard solution. Intraday precision and interday precision were assessed with CV values calculated after measurements for within day and 3 consecutive days on the three concentrations, respectively. The CV (%) value was calculated from Equation (2).

\[
\text{CV} (\%) = \frac{\text{standard deviation} (SD)}{\text{mean}} \times 100 \quad (2)
\]

3.7. Statistical Analysis

Data were expressed as mean, SD, and CV (%) using Microsoft Excel 2019 software (Microsoft Co., Redmond, WA, USA).

4. Conclusions

In this study, for the first time, a sensitive, accurate, and reliable LC–MS/MS MRM assay for efficient quality assessment of GHT, a traditional herbal prescription, was developed using nine selected marker analytes. The developed assay was evaluated by examining the linearity, LOD, LOQ, accuracy, and precision. The established LC–MS/MS MRM assay is expected to be useful in not only the efficient quality control of GHT, but also in further studies on other TKMs, TCMs, and KMs.

Supplementary Materials: The following supporting information can be downloaded, Figure S1: Chemical structures of the nine marker components in GHT; Figure S2: MS fragmentation of each marker analyte; Figure S3: Extracted ion chromatograms of each reference standard (A) and GHT sample (B) measured by LC–MS/MS MRM mode; Table S1: Composition of prepared GHT; Table S2: Repeatability of the nine marker analytes in the LC–MS/MS MRM assay (n = 6); Table S3: LC–MS/MS MRM experimental conditions for simultaneous determination of the nine marker analytes in GHT samples.

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References
1. Mao, Q.; Xu, J.; Kong, M.; Shen, H.; Zhu, H.; Zhou, S.; Li, S. LC-MS-based metabolomics in traditional Chinese medicines research: Personal experiences. Chin. Herb. Med. 2017, 9, 14–21. [CrossRef]
2. Li, S.L.; Song, J.Z.; Qiao, C.F.; Zhou, Y.; Xu, H.X. UPLC-PDA-TOF-MS based chemical profiling approach to rapidly evaluate chemical consistency between traditional and dispensing granule decoctions of traditional medicine combinatorial formulae. J. Pharm. Biomed. Anal. 2010, 52, 468–478. [CrossRef] [PubMed]
3. Wu, T.Y.; Chang, F.R.; Liou, J.R.; Lo, I.W.; Chung, T.C.; Lee, L.Y.; Chi, C.C.; Du, Y.C.; Wong, M.H.; Juo, S.H.H.; et al. Rapid HPLC quantification approach for detection of active constituents in modern combinatorial formula, San-Huang-Xie-Xin-Tang (SHXXT). Front. Pharmacol. 2016, 7, 574. [CrossRef] [PubMed]
4. An, S.H.; Shin, H.R.; Park, K.; Lee, Y.S.; Kim, J.; Yeom, S.R.; Kwon, Y.D.; Cho, H.Y. Safety of Gunghatang tablet after single oral administration in healthy male volunteers, single center study. J. Korean Med. Rehabil. 2019, 29, 101–108.
5. Heo, J.; Donguibogam: Namsandang. Seoul, Korea, 2007; p. 134.
6. Kim, H.G.; Oh, S.M.; Kim, N.W.; Shim, J.H.; Nam, Y.H.; Nguyen, T.N.; Lee, M.H.; Lee, D.Y.; Kang, D.H.; Baek, N.I. Three new phthalide glycosides from the rhizomes of *Cnidium officinale* and their recovery effect on damaged otic hair cells in zebrafish. *Molecules* 2021, 26, 7034. [CrossRef]
7. Li, Y.; Li, D.; Chen, J.; Wang, S. A polysaccharide from *Pinellia ternata* inhibits cell proliferation and metastasis in human cholangiocarcinoma cells by targeting of Cdc42 and 67 kDa laminin receptor (LR). *Int. J. Biol. Macromol.* 2016, 93, 520–525. [CrossRef]
8. Tu, Y.; Luo, X.; Liu, D.; Li, H.; Xia, H.; Ma, C.; Zhang, D.; Yang, Y.; Pan, X.; Wang, T.; et al. Extracts of *Poria cocos* improve functional dyspepsia via regulating brain–gut peptides, immunity and repairing of gastrointestinal mucosa. *Phytochemistry* 2021, 95, 153875. [CrossRef]
9. Jang, A.; Choi, G.E.; Kim, Y.J.; Lee, G.H.; Hyun, K.Y. Neuroprotective properties of ethanolic extract of *Citrus unshiu* Markovich peel through NADPH oxidase 2 inhibition in chemotherapy-induced neuropathic pain animal model. *Phytother. Res.* 2021, 35, 6918–6931. [CrossRef]
10. Ahn, T.S.; Hwang, D.S.; Lee, J.M.; Jang, J.B.; Lee, C.H. Anti-inflammatory effect and mechanism of *Citri Reticulatae Viride* peel through NADPH oxidase 2 inhibition in chemotherapy-induced neuropathic pain animal model. *Molecules* 2022, 27, 1223. [CrossRef]
11. Okada, N.; Murakami, A.; Urushizaki, S.; Matsuda, M.; Kawazoe, K.; Ishizawa, K. Extracts of immature orange (*Aurantii fructus immatarus*) and *Citrus Unshiu* peel (*Citrus unshiu pericarpium*) induced p-glucoprotein and cytotoxome P450 3A4 expression via upregulation of pregnane X receptor. *Front. Pharmacol.* 2017, 8, 84. [CrossRef]
12. Eun, S.Y.; Cheon, Y.H.; Park, K.D.; Chung, C.H.; Lee, C.H.; Kim, J.Y.; Lee, M.S. Anti-osteoporosis effects of the *Eleutherococcus senticosus*, *Achyranthes japonica*, and *Atractylodes japonica* mixed extract fermented with nuruk. *Nutrients* 2021, 13, 3904. [CrossRef] [PubMed]
13. Liu, H.; Cui, J.; Zhang, L.; Chang, G.; Wang, W. Screening of anti-chronic nonbacterial prostatitis activity of different extractions of the aerial part of *Glycyrrhiza uralensis*, and network pharmacology research. *Biomed. Rep.* 2021, 15, 99. [CrossRef] [PubMed]
14. Abbas, A.N. Ginger (*Zingiber officinale* (L.) Rosc) improves oxidative stress and trace elements status in patients with alopecia areata. *Niger. J. Clin. Pract.* 2020, 23, 1555–1560. [CrossRef] [PubMed]
15. Baek, M.E.; Seong, G.U.; Lee, Y.J.; Won, J.H. Quantitative analysis for the quality evaluation of active ingredients in *Cnidium Rhizome*. *Yakhwak Hoeji* 2016, 60, 227–234. [CrossRef]
16. 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 ternata* by various processing method and its safety estimate. *Korean J. Orient. Physiol. Pathol.* 2004, 18, 846–853.
17. Wu, L.F.; Wang, K.F.; Mao, X.; Liang, W.Y.; Chen, W.J.; Li, S.; Qi, Q.; Cui, Y.P.; Zhang, L.Z. Screening and analysis of the potential bioactive components of *Poria cocos* (Schw.) Wolf by HPLC and HPLC-MS<sup>8</sup> with the aid of chemometrics. *Molecules* 2016, 21, 227. [CrossRef]
18. Liu, E.H.; Zhao, P.; Duan, L.; Zheng, G.D.; Guo, L.; Yang, H.; Li, P. Simultaneous determination of six bioactive flavonoids in *Citri Reticulatae Pericarpium* by rapid resolution liquid chromatography coupled with triple quadrupole electrospray tandem mass spectrometry. *Food Chem.* 2013, 141, 3977–3983. [CrossRef]
19. Kim, H.G.; Kim, G.S.; Lee, J.H.; Park, S.; Jeong, W.Y.; Kim, Y.H.; Kim, J.H.; Cho, Y.A.; Lee, W.S.; et al. Determination of the change of flavonoid compounds as the defence materials of *Citrus unshiu* Marc. fruit peel against *Penicillium digitatum* by liquid chromatography coupled with tandem mass spectrometry. *Food Chem. 2011*, 128, 49–54. [CrossRef]
20. Wang, C.; Pan, Y.; Fan, G.; Chai, Y.; Wu, Y. Application of an efficient strategy based on MAE, HPLC-DAD-MS/MS and HSCCC for the rapid extraction, identification, separation and purification of flavonoids from Fructus Aurantii Immaturus. *Biomed. Chromatogr.* 2010, 24, 235–244. [CrossRef]
21. Chen, Q.; He, H.; Li, P.; Zhu, J.; Xiong, M. Identification and quantification of atracylenolide I and atracylenolide III in Rhizoma *Atractylodes macrocephala* by liquid chromatography-ion trap mass spectrometry. *Biomed. Chromatogr.* 2013, 27, 699–707. [CrossRef]
22. Wu, Y.P.; Meng, X.S.; Bao, Y.R.; Wang, S.; Kang, T.G. Simultaneous quantitative determination of nine active chemical compositions in traditional Chinese medicine *Glycyrrhiza* by RP-HPLC with full-time five-wavelength fusion method. *Am. J. Chin. Med.* 2013, 41, 211–219. [CrossRef] [PubMed]
23. Ali, G.; Hawa, Z.E.J.; Ali, B.; Amin, T.M. Formation of shogaol in ginger through application of different methods: Altered antioxidant and antimicrobial activity. *Molecules* 2018, 23, 1646.
24. Seo, C.S.; Shin, H.K. Development and validation of a high-performance liquid chromatography method for quality assessment of oriental medicine, Dokhwalsasaeng-tang. *Appl. Sci.* 2021, 11, 7829. [CrossRef]
25. Xie, M.; Yu, Y.; Zhi, Z.; Deng, L.; Ren, B.; Zhang, M. Simultaneous determination of six main components in Bushen Huoxue prescription by HPLC-CAD. *J. Pharm. Biomed. Anal.* 2021, 201, 114087. [CrossRef]
26. Wen, X.; Luo, K.; Xiao, S.; Ai, N.; Wang, S.; Fan, X. Qualitative analysis of chemical constituents in traditional Chinese medicine analogous formula cheng-Qi decoctions by liquid chromatography–mass spectrometry. *Biomed. Chromatogr.* 2016, 30, 301–311. [CrossRef]
27. Wang, L.; Wu, C.; Zhao, L.; Lu, X.; Wang, F.; Yang, J.; Xiong, Z. An ultra-performance liquid chromatography photodiode array detection tandem mass spectrometric method for simultaneous determination of seven major bioactive constituents in *Xiaoaihuatang* and its application to fourteen compatibilities study. *J. Chromatogr. Sci.* 2015, 53, 1570–1576. [CrossRef]
28. Wang, L.; Wu, C.; Zhao, L.; Lu, X.; Wang, F.; Yang, J.; Xiong, Z. An ultra-performance liquid chromatography photodiode array detection tandem mass spectrometric method for simultaneous determination of seven major bioactive constituents in *Xiaoaihuatang* and its application to fourteen compatibilities study. *J. Chromatogr. Sci.* 2015, 53, 1570–1576. [CrossRef]
28. He, M.; Yang, Z.Y.; Yang, T.B.; Ye, Y.; Nie, J.; Hu, Y.; Yan, P. Chemometrics-enhanced one-dimensional/comprehensive two-dimensional gas chromatographic analysis for bioactive terpenoids and phthalides in Chaihu Shugan San essential oils. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2017, 1052, 158–168. [CrossRef]

29. Wang, X.; Zhang, A.; Yan, G.; Han, Y.; Sun, H. UHPLC-MS for the analytical characterization of traditional Chinese medicines. Trends Anal. Chem. 2014, 63, 180–187. [CrossRef]

30. Ying, X.; Liu, M.; Liang, Q.; Jiang, M.; Wang, Y.; Huang, F.; Xie, Y.; Shao, J.; Bai, G.; Luo, G. Identification and analysis of absorbed components and their metabolites in rat plasma and tissues after oral administration of ‘Ershuweiqi Shanhui’ pill extracts by UPLC-DAD/Q-TOF-MS. J. Ethnopharmacol. 2013, 150, 324–338. [CrossRef]

31. Fu, C.; Xia, Z.; Liu, Y.; Lu, H.; Zhang, Z.; Wang, Y.; Fan, X. Qualitative analysis of major constituents from Xue Fu Zhu Yu Decoction using ultra high performance liquid chromatography with hybrid ion trap time-of-flight mass spectrometry. J. Sep. Sci. 2016, 39, 3457–3468. [CrossRef]

32. Wang, Y.; Yang, L.; He, Y.Q.; Wang, C.H.; Welbeck, E.W.; Bligh, S.W.; Wang, Z.T. Characterization of fifty-one flavonoids in a Chinese herbal prescription Longdan Xiegan Decoction by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry and photodiode array detection. Rapid Commun. Mass Spectrom. 2008, 22, 1767–1778. [CrossRef] [PubMed]

33. Zheng, G.D.; Zhou, P.; Yang, H.; Li, Y.; Li, P.; Liu, E.H. Rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry method for identification of chemical constituents in Citri Reticulatae Pericarpium. Food Chem. 2013, 136, 604–611. [CrossRef] [PubMed]

34. Tan, G.; Zhu, Z.; Jing, J.; Lv, L.; Lio, Z.; Zhang, G.; Chai, Y. Characterization of constituents in Sini decoction and rat plasma by high-performance liquid chromatography with diode array detection coupled to time-of-flight mass spectrometry. Biomed. Chromatogr. 2011, 25, 913–924. [CrossRef] [PubMed]

35. Tao, Y.; Li, W.; Liang, W.; Van Breemen, R.B. Identification and quantification of gingerols and related compounds in ginger dietary supplements using high performance liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 2009, 57, 10014–10021. [CrossRef]

36. Stafiński, M.; Wieczorek, M.; Janicki, P.; Kościelniak. Theoretical and experimental examination of recovery in the context of trueness of analytical results. Talanta 2012, 96, 39–43. [CrossRef]

37. Stafiński, M.; Wieczorek, M.; Janicki, P.; Kościelniak. Influence of the species effect on trueness of analytical results estimated by the recovery test when determining selenium by HG-AFS. Talanta 2013, 117, 64–69. [CrossRef]

38. Seo, C.S.; Lee, M.Y. Simultaneous quantification of eight marker components in traditional herbal formula, Haepyoyijin-tang using HPLC–PDA. Appl. Sci. 2020, 10, 3888. [CrossRef]

39. 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]

40. Seo, C.S.; Shin, H.K. Liquid chromatography tandem mass spectrometry for the simultaneous quantification of eleven phytochemical constituents in traditional Korean medicine, Sogunjun decoction. Processes 2021, 9, 153. [CrossRef]