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Simultaneous Determination of 11 Marker Compounds in Gumiganghwal-tang by HPLC-DAD and LC-MS

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Abstract – Gumiganghwal-tang has been used for the treatment of common cold for a long-time. We developed an accurate and sensitive high performance liquid chromatography-diode array detection (HPLC-DAD) and electrospray ionization mass spectrometry method for the simultaneous determination of ferulic acid, baicalin, bergapten, methyl eugenol, glycyrrhizin, oxypeucedanin, wogonin, nodakenin, atractylenolide III, imperatorin, and atractylenolide I in Gumiganghwal-tang samples. The analytes were separated on a Shiseido C18 column (5 μm, 4.6 mm I.D. × 250 mm) with gradient elution with acetonitrile and 0.1% trifluoroacetic acid. Eleven compounds were quantitatively determined by HPLC-DAD and identified by LC-MS data. We also validated this method. The calibration curves of all the compounds showed good linear regression. The limits of detection and the limits of quantification ranged from 0.04 to 0.63 and from 0.12 to 1.92 μg/mL, respectively. The relative standard deviation values of intra- and inter-days of this method represented less than 2.9%. The recoveries were found to be in the range of 90.06 – 107.66%. The developed method has been successfully applied to the analysis of Gumiganghwal-tang samples. The established HPLC method could be used to quality control of Gumiganghwal-tang.
Keywords – Simultaneous determination, Gumiganghwal-tang; HPLC-DAD, LC-MS

Introduction

For centuries traditional herbal medicines and their prescriptions are used for the prevention and treatment of various diseases in many oriental countries and others, Korea, China, and Japan. These herbal medicines have only few side effects and, as single components, these exhibit therapeutic properties for multiple diseases.[1] Moreover, multiple constituents of herbal prescriptions might have synergic effects.[2] Therefore, herbal products have gained increasing popularity, and "herbal medicine has become a popular form of healthcare.[3,4]

Gumiganghwal-tang is one of the Korean traditional preparations that is recorded in the traditional medicine book, Dong-Ui-Bo-Gam (Medical Thesaurus of Korea), published in 1613. This prescription has been used for the treatment of common cold, headache, arthralgia, and fever. It also exhibits anti-inflammatory, analgesic, and antipyretic activities.[5,6] Furthermore, the no-observed-adverse-effect-level for Gumiganghwal-tang orally administered to rats was determined to be over 2000 mg/kg/day for both genders.[7]

Gumiganghwal-tang is composed of 9 crude drugs: *Ostericum koreanum* Maximowicz, *Saposhnikovia divaricata* Schiskin, *Cnidium officinale* Makino, *Angelica dahurica* Bentham et Hooker, *Atractylodes japonica* Koidzumi, *Scutellaria baicalensis* Georgi, *Rehmannia glutinosa* Liboschitz var, *Asiasarum sieboldi* F. Maekawa, and *Glycyrrhiza uralensis* Fischer. Among all the others, *O. koreanum* and *S. divaricate*, which have similar properties, are the most important crude drugs. *Ostericum koreanum* exhibits anti-allergic, anti-inflammatory, and anti-tumor activities.[8-10] *Saposhnikovia divaricata* also has anti-allergic and anti-inflammatory properties.[11,12] Moreover, both of them have analgesic and antifebrile activity.

The contents of bioactive compounds in traditional herbal medicines such as Gumiganghwal-tang depend on plant origin, cultivation sites, harvest seasons, and other factors,[13] and various interactions between the bioactive components of the used herbs can occur during the manufacturing process.[14] Accurate quantitative and qualitative analysis data of bioactive compounds in traditional herbal

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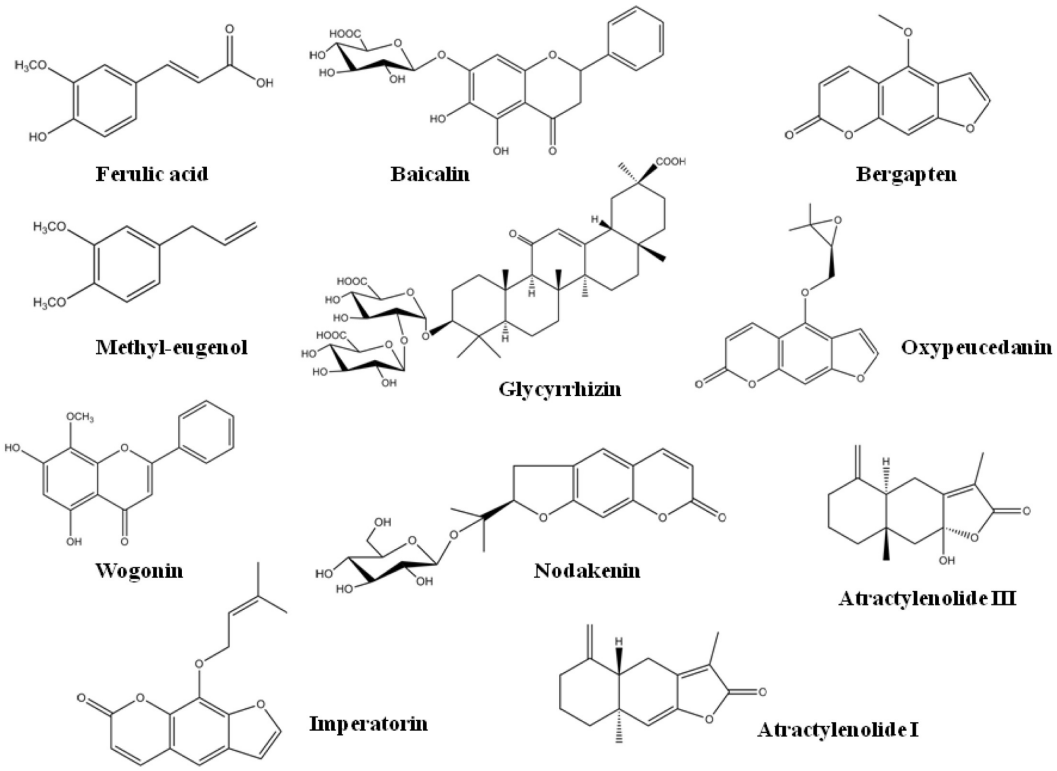

Fig. 1. Chemical structures of the 11 standard compounds of Gumiganghwal-tang.

medicine can be used to evaluate herbal prescriptions. Therefore, it seems to be necessary to determine a reliable and accurate method for quality control of herbal medicines.

However, analytical determination methods for the quality control of these compounds have not been developed yet. The objective of this research was to investigate the contents of compounds present in Gumiganghwal-tang.

In this study, the accurate and reliable high performance liquid chromatography-diode array detection (HPLC-DAD) method for the simultaneous determination of the 11 compounds (i.e., ferulic acid of *C. officinale*, baicalin and wogonin of *S. baicalensis*, bergapten and imperatorin of *S. divaricate* and *A. dahurica*, methyl eugenol of *A. sieboldi*, glycyrrhizin of *G. uralensis*, oxypeucedanin and nodakenin of *O. koreanum* and atractylenolide III and atractylenolide I of *A. japonica*) in Gumiganghwal-tang were developed and validated (Fig. 1). These marker compounds were selected on the basis of the amount present in each crude drug and its therapeutic effects.[5]

Previous studies reported that HPLC analysis method of Xiaochaihu Tang, Huangqin-Tang and sann-joong-kuey-jian-tang including baicalin, wogonin and glycyrrhizic acid for simultaneous determination was established.[15-17]

We additionally identified the 11 marker compounds using liquid chromatography-mass spectrometry (LC-MS).

Experimental

Instruments – The HPLC equipment used was a Dionex system (Dionex, Germany) composed of a pump (LPG 3X00), an auto sampler (ACC-3000), a column oven (TCC-3000SD), diode array UV/VIS detector [DAD-3000(RS)] and Dionex Chromeleon Chromatography Data System software. HPLC analysis was conducted on a Shiseido C_{18} column (4.6 mm I.D. × 250 mm, 5-μm pore size).

The LC-MS system used was a TSQ Quantum Ultra (Thermo Electron Co., USA) coupled with electro-spray ionization in positive-ion mode in the Central Laboratory of Kangwon National University. Standard and sample solutions were separated on the Shiseido C_{18} column used for the HPLC analysis.

Chemicals and reagents – Imperatorin, ferulic acid, and bergapten were purchased from Sigma Aldrich Co. Ltd (USA). Oxypeucedanin, nodakenin, atractylenolide I, atractylenolide III, baicalin, wogonin, methyl eugenol, and glycyrrhizin were purchased from Korea Food and Drug Administration. The purities of the 11 standards were above 98%. HPLC-grade acetonitrile, methanol, and water

were purchased from J. T. Baker (USA). Trifluoroacetic acid (TFA) was purchased from DAE JUNG (Korea). Twelve commercial Gumiganghwal-tang samples as powder were provided by the Korea Institute of Oriental Medicine.

Preparation of standard and sample solutions – Standard stock solutions of imperatorin (220 μg/mL), oxypeucedanin (220 μg/mL), nodakenin (96 μg/mL), ferulic acid (100 μg/mL), bergapten (410 μg/mL), atractylenolide III (100 μg/mL), atractylenolide I (100 μg/mL), baicalin (660 μg/mL), wogonin (460 μg/mL), methyl eugenol (260 μg/mL), and glycyrrhizin (200 μg/mL) were prepared in methanol. These standard stock solutions were mixed and diluted to 6 different concentrations by methanol to establish the calibration curves.

These standard stock solutions also mixed and diluted to 3 different concentrations to validate method in precision and accuracy test.

Gumiganghwal-tang samples (GMGH 1-12) were weighed and dissolved in distilled water at 20.92, 20.14, 20.12, 20.04, 20.12, 20.08, 20.00, 20.00, 20.00, 20.00, 20.00, and 20.00 mg/mL, respectively. Before HPLC analysis, all the sample solutions were filtered through a 0.45-μm membrane filter and stored in a refrigerator at below 4 ºC.

Liquid chromatography conditions – The mobile phase consisted of 0.1% TFA aqueous solution (A) and acetonitrile (B) operating at a flow rate of 1.0 mL/min. The HPLC linear gradient profile was as follows: 10% B at 0 min, 10 – 30% B at 0 – 15 min, 30% B at 15 – 30 min, 30 – 37% B at 30 – 35 min, 37 – 40% B at 35 – 40 min, 40% B at 40 – 45 min, and 40 – 90% B at 45 – 60 min. The injection volume was 20 μL. The column temperature was set at 35 ºC. For the determination of each standard compounds, 4 different UV spectra were selected: 205 nm, 250 nm, 280 nm, and 330 nm.

LC-MS conditions – LC-MS was used for the identification of each compound peak in standard and sample solutions. The analysis was performed under the same column, mobile phases without TFA and gradient system used for HPLC-DAD analysis. The injection volume was 20 μL. Positive-ion electro-spray ionization was performed at 4000 V spray voltage. The vaporizer and capillary temperatures were maintained at 100 ºC and 350 ºC, respectively. The sheath gas pressure and aux gas pressure were set at 60 psi and 30 psi, respectively. Selected ion monitoring (SIM) was used for MS spectra scan.

Validation of the HPLC method – The established HPLC method was validated according to the International Conference on Harmonisation guidelines. The validation was performed in terms of linearity, precision, and accuracy.[18-21]

Quantification of Gumiganghwal-tang samples – The established method was applied to analyze 12 Gumiganghwal-tang samples, and each analysis was conducted in triplicate. The quantification of 11 marker compounds in commercial Gumiganghwal-tang samples was performed using the relative calibration curves.

Result and Discussion

Optimization of HPLC-DAD condition – To develop this simultaneous determination method, 11 standard compounds from different crude plants were selected. Moreover, to obtain chromatograms with good separation, column type, column temperature, mobile phase, and detection wavelength were carefully investigated. Various columns were compared to obtain the optimal analysis conditions: Dionex C_{18} column (150 mm × 4.6 mm I.D., 5 μm), LUNA C_{18} column (250 × 4.6 mm I.D., 5 μm,), and Shiseido C_{18} column (250 × 4.6 mm I.D., 5 μm). Among these, the Shiseido C_{18} column exhibited the best resolution. Resolution value ≥ 1.5 represents good peak resolution in many cases. Resolution value of oxypeucedanin was 1.52. However, resolution value of others column was lower than 1.5. The gradient system, column temperature (30 ºC, 35 ºC, and 40 ºC), and flow rate were optimized to obtain a good resolution of the compounds. As a result, the optimal conditions as described in "Liquid chromatography conditions" session were established. TFA (0.1% in water) was added to the mobile phase to obtain the inhibition of peak tailing and to improve the peak shape. Because of the different responses of each standard compound to the detection wavelength, various UV absorption wavelengths were investigated. As a result, according to the maximum absorption values of compounds obtained with the DAD system, 4 UV wavelengths were selected: 205 nm for imperatorin, nodakenin, bergapten, atractylenolide III, wogonin, and methyl eugenol; 250 nm for oxypeucedanin and glycyrrhizin; 280 nm for atractylenolide I and baicalin; and 330 nm for ferulic acid (Fig. 2). The HPLC chromatogram of the standard compounds is shown in Figure 3A. The retention time of ferulic acid, nodakenin, baicalin, bergapten, glycyrrhizin, oxypeucedanin, wogonin, methyl eugenol, atractylenolide III, imperatorin, and atractylenolide I were 16.23, 16.84, 19.48, 36.17, 42.70, 43.99, 44.78, 47.84, 49.83, 53.86, and 58.68 min, respectively. The identity of each peak of 11 compounds in Gumiganghwal-tang sample was confirmed by comparing the peak retention time and UV

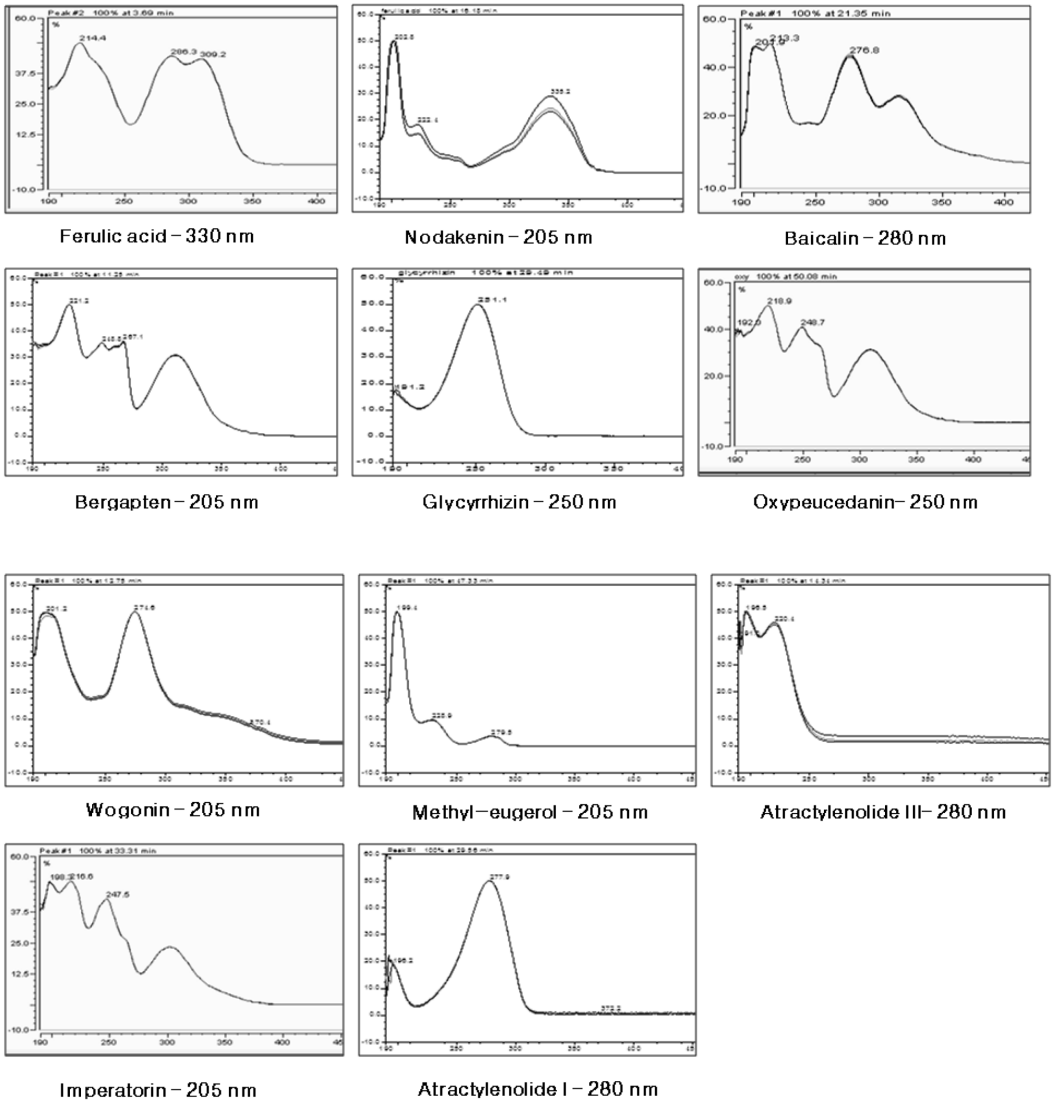

Fig. 2. UV wavelength of the 11 standard compounds of Gumiganghwal-tang.

spectrum of standard compounds.

Linearity, limits of detection, and limits of quantification – Calibration curves were plotted for each standard compound, and the relative regression coefficients (R^2) were calculated to validate their linearity. The standard stock solution containing 11 marker compounds was diluted into 6 appropriate concentrations to plot the calibration curves. Each diluted standard solution was analyzed in triplicate. The calibration curves were constructed using the peak areas (y) and the concentration of analytes (x) by using regression equations in the form of $y = ax + b$. The limits of detection (LOD) and limits of quantification (LOQ) values were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively (Table 1). All the calibration data of the 11 standard compounds showed good linearity ($R^2 > 0.9970$) in a relatively wide concentration range. The LOD and LOQ values of all standard compounds were in the range 0.06 – 0.63 and 0.12 – 1.92 μg/mL, respectively.

Precision and accuracy – The precision of the developed method was estimated by intra- and inter-day variations, and it was expressed by the relative standard deviation (RSD). RSD was calculated by measuring the standard deviation over the mean of the measured amount and multiplying that value by 100. The RSD values of intra- and inter-day were 0.12 – 2.90% and 0.01 – 2.38%, respectively. The intra-day accuracy was in the range of 90.21 – 109.63% and the inter–day accuracy was 90.32 – 109.76%. The results of the intra- and inter-day tests are shown in Table 2.

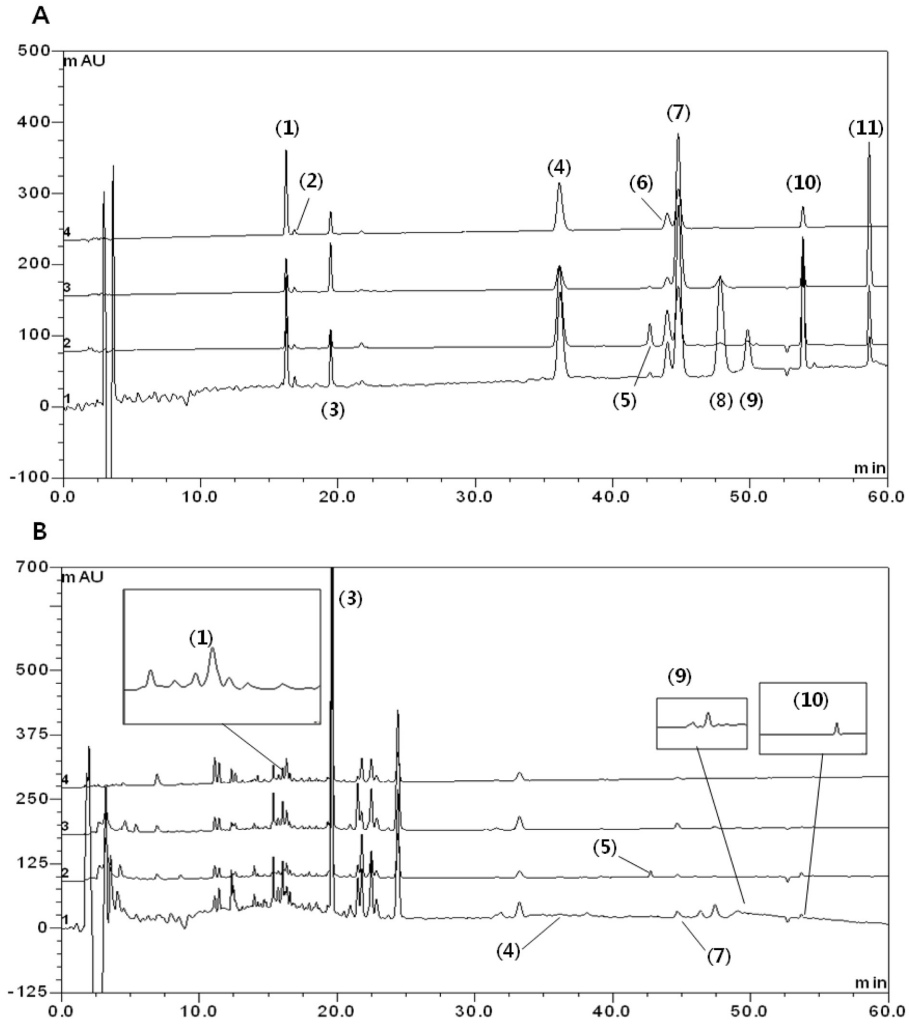

Fig. 3. HPLC chromatogram of standard compounds mixture (A) and Gumiganghwal -tang sample (B). Peaks: (1) ferulic acid, (2) nodakenin, (3) baicalin, (4) bergapten, (5) glycyrrhizin, (6) oxypeucedanin, (7) wogonin, (8) methyl eugenol, (9) atractylenolide III, (10) imperatorin and (11) atractylenolide I.

To assess the accuracy of the method, the recovery test of the 11 marker compounds was performed by the standard addition method. Three different concentrations of mixed standard solution were added to the Gumiganghwal-tang sample and analyzed in triplicate. The recovery was calculated by the equation: (amount found – original amount) / (amount spiked) × 100. The overall recoveries were in the range of 90.06–107.66%, and the RSD values were measured from 0.15% to 2.41% (Table 3). These results demonstrate that this method has a suitable precision and accuracy for the simultaneous determination of the compounds in Gumiganghwal-tang.

LC-MS analysis – The identification of 11 marker compounds in Gumiganghwal-tang was also carried out by LC-MS analysis. We analyzed individually standard compound before sample analysis. Comparing the peak retention time and mass spectra of standard compounds, 11 compounds in sample were identified.

Under the same conditions of the HPLC analysis, all the 11 marker compounds (i.e., ferulic acid, baicalin, bergapten, methyl eugenol, glycyrrhizin, oxypeucedanin, wogonin, nodakenin, atractylenolide III, imperatorin, and atractylenolide I) were clearly detected (Fig. 4). The peak observed at m/z 823.47 corresponded to the $[M+H]^+$ of glycyrrhizin ($C_{42}H_{62}O_{16}$, molecular weight [MW] = 822.93 g/mol). Moreover, ferulic acid ($C_{10}H_{10}O_4$, MW = 194.18 g/mol), baicalin ($C_{21}H_{18}O_{11}$, MW = 446.36 g/mol), bergapten ($C_{12}H_8O_4$, MW = 216.19 g/mol), methyl eugenol ($C_{11}H_{14}O_2$, MW = 178.23 g/mol), oxypeucedanin ($C_{16}H_{14}O_5$, MW = 286.28 g/mol), wogonin ($C_{16}H_{12}O_5$, MW = 284.26 g/mol), nodakenin ($C_{20}H_{24}O_9$, MW = 408.40 g/mol), atractylenolide III ($C_{15}H_{20}O_3$, MW = 248.32 g/mol), imperatorin ($C_{16}H_{14}O_4$,

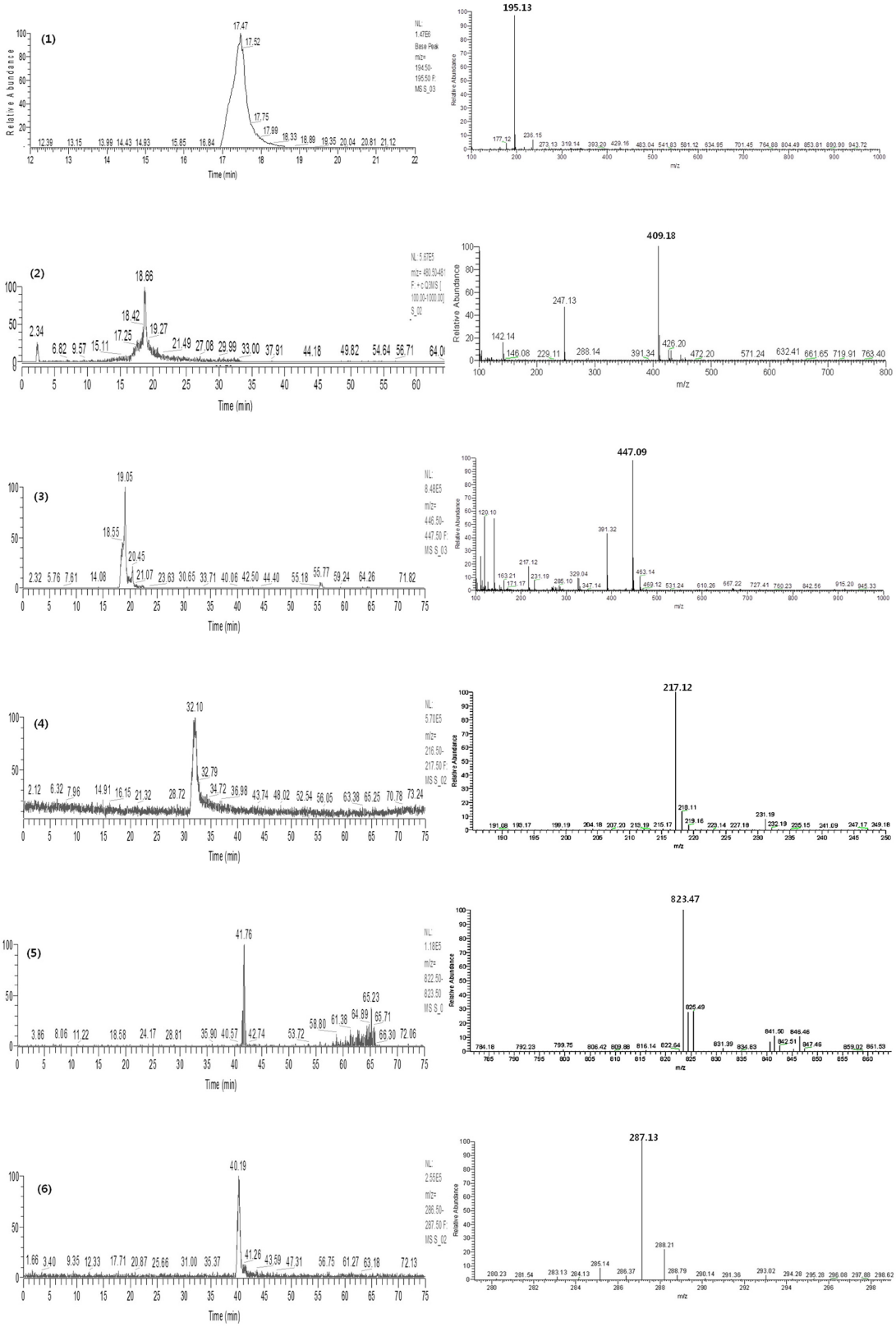

Fig. 4. SIM chromatograms and product ion scan spectra of the 11 marker compounds of Gumiganghwal-tang. Numbers: (1) ferulic acid, (2) nodakenin, (3) baicalin, (4) bergapten, (5) glycyrrhizin, (6) oxypeucedanin, (7) wogonin, (8) methyl eugenol, (9) atractylenolide III, (10) imperatorin and (11) atractylenolide I.

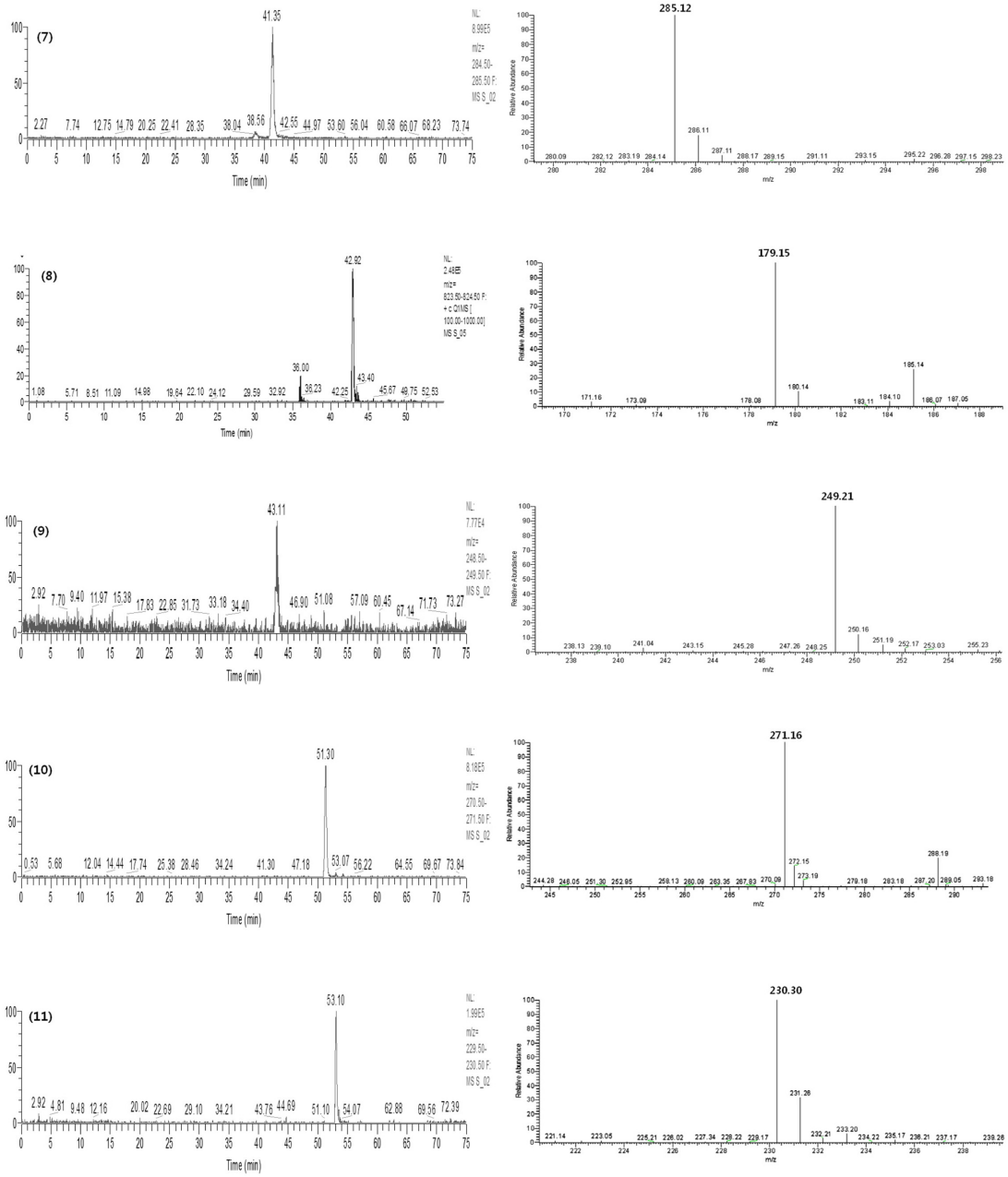

Fig. 4. continued.

MW = 270.28 g/mol), and atractylenolide I ($C_{15}H_{18}O_2$, MW = 230.30 g/mol) were also detected as $[M+H]^+$ or $[M]^+$ (Table 4).

Application of the method to real Gumiganghwal-tang sample analysis – The developed HPLC-DAD method was applied for determination of the 11 standard compounds in 12 commercial Gumiganghwal-tang samples. The amounts of the 11 standard compounds were calculated (Table 5).

The established method showed accurate separation of the Gumiganghwal-tang samples without any effect of the other peaks (Fig. 3B). The amounts of all marker compounds in the 12 commercial Gumiganghwal-tang samples were generally similar. The concentrations of ferulic acid, glycyrrhizin, wogonin, atractylenolide III and imperatorin were in the ranges 0.253 – 0.338, 0.375 – 1.176, 0.043 – 0.138, 0.036 – 0.082 and 0.041 – 0.150 μg/mg, respectively. Among the 11 marker compounds, baicalin was present in the highest concentration (17.893 – 22.086 μg/mg). The peak corresponding to bergapten was

detected in several samples, but in most cases, the amount of this component was lower than its LOQ value. Furthermore, methyl eugenol, oxypeucedanin, nodakenin and atractylenolide I were not detected in all the samples. The variations of these components could be due to different environments or manufacturing processes of commercial Gumiganghwal-tang.

Simultaneous determination of 11 marker compounds, ferulic acid, baicalin, bergapten, methyl eugenol, glycyrrhizin, oxypeucedanin, wogonin, nodakenin, atractylenolide III, imperatorin, and atractylenolide I was developed for quality control of Gumiganghwal-tang and validated by linearity, precision and accuracy. As result of validation, this developed method showed good linearity, precision and recovery. The validation data indicated that this HPLC method is accurate and reliable.

Based on previous HPLC analysis method of Xiaochaihu Tang, Huangqin-Tang and sann-joong-kuey-jian-tang, we set new mobile phase with gradient system and detected wavelength of baicalin, wogonin and glycyrrhizic acid. In this study, LC-MS is additionally used for qualitative analysis of compounds. Therefore, this method is sensitive analysis method than previous reported analysis method.

Moreover, this method was successfully applied for the quantitative analysis of 11 compunds in Gumiganghwal-tang and analysis data confirmed that contents of the 11 major compounds is different due to cultural environment, such as area and climate and manufacturing processes. This established method is considered suitable quality control for Gumiganghwal-tang.

The developed HPLC method for the simultaneous determination of 11 marker compounds, ferulic acid, baicalin, bergapten, methyl eugenol, glycyrrhizin, oxypeucedanin, wogonin, nodakenin, atractylenolide III, imperatorin, and atractylenolide I in Gumiganghwal-tang was successfully analyzed. Previously, analytical HPLC method has been applied for determination of Gumiganghwal-tang.[22] However, referred method analyzed fewer compounds in Gumiganghwal-tang and method validation was insufficient to apply to quality control. In this study, the established method could be successfully applied to quantitatively analyze the 11 marker compounds found in commercial Gumiganghwal-tang samples and that it is suitable for quality evaluations. The established method could be used to improve the quality of Gumiganghwal-tang.

Acknowledgments

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