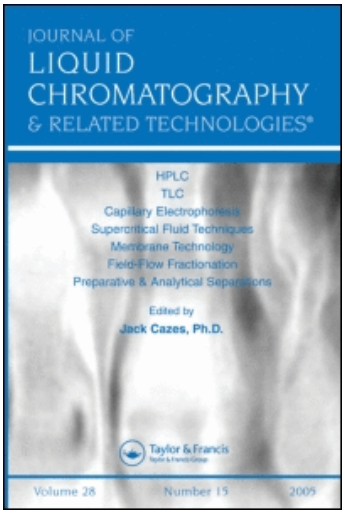

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QUALITATIVE AND QUANTITATIVE ANALYSIS OF 15 ACTIVE CONSTITUENTS IN JIWEILING FREEZE-DRIED POWDER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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QUALITATIVE AND QUANTITATIVE ANALYSIS OF 15 ACTIVE CONSTITUENTS IN JIWEILING FREEZE-DRIED POWDER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Minyan Liu,[1,2] Shaohua Zhao,[2] Jiming Jia,[2] Xiaowei Shi,[3] Jian Song,[2] Hongtao Wang,[2] Yingfeng Du,[1] and Lantong Zhang[1]

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□ *Qualitative characterization and quantitative analysis of 15 bioactive constituents in Jiweiling freeze-dried powder (JWL) have been achieved by reversed-phase high performance liquid chromatography coupled with tandem mass spectrometry, including five flavonoids and ten ginsenosides. Chromatographic separation was performed on an Agilent ZORBAX Eclipse XDB-C_{18} HPLC column, with gradient elution of 0.1% formic acid aqueous solution and acetonitrile as mobile phase. The compounds were detected in the negative ion mode with multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer equipped with an electrospray ionization interface. By using of this method, 15 bioactive components were unequivocally identified on the basis of their characteristic fragmentation patterns. All calibration curves showed good linearity ($r^2 > 0.998$) within the test ranges. The LOD, LOQ, specificity, precision, and accuracy for the method were validated. The proposed method was successfully applied to analyze the quality of 12 batches of JWL. The results indicated that this analytical method was simple and suitable for the identification and quality assessment of JWL.*

Keywords fifteen constituents, HPLC-ESI-MS/MS, Jiweiling freeze-dried powder, MRM, qualitative characterization, quantitative analysis

INTRODUCTION

Jiweiling freeze-dried powder (JWL) is a compound preparation developed with Chinese herb extracts of *Panax ginseng* and *Epimedium*,

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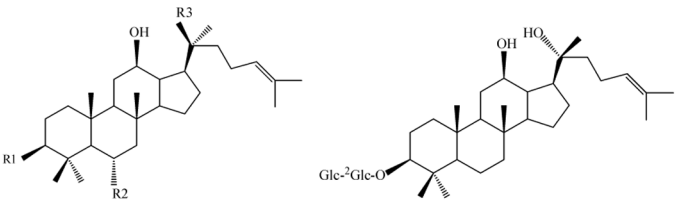
which is responsible for motor neuron diseases (MNDs). MNDs are a rare group of progressive neurological diseases,[1] which involve the degeneration of motor neurons, leading to progressive muscle weakness, atrophy, pain, paralysis, oropharyngeal dysfunction, respiratory impairment, and, ultimately, death within 3 to 5 years after disease onset.[1–5] There are no effective treatments and drugs for MND because the causes of MND remain unknown in modern medicine. Western medicine Rilutek, the only FDA-approved drug for MND, can delay the process of amyotrophic lateral sclerosis (ALS) and prolong the lifespan of ALS patients, but has little effects on the symptoms, especially for the oropharyngeal dysfunctions. Moreover, there are some adverse reactions: asthenia, spasticity, mild elevations in aminotransferase levels, and so on.[6,7] Traditional Chinese medicines (TCMs) and their products have been attracting more and more attention because of their unique therapeutic effects on delaying the disease process and improving the symptoms.[8] JWL had been used to treat the patients with MNDs for many years in clinical as a hospital preparation and had achieved good efficacy.[9] Several studies had been reported that JWL could decrease the apoptosis of motoneuron induced by glutamate, promote primary cultured motoneurons of rat spinal cord growth and protect the normal and excitatory amino acids injury motoneuron.[10–12] Experiments from pharmacological laboratory in USA demonstrated that JWL could significantly prolong the survival time of ALS transgenic mouse model and delay the paralyzed time, particularly the appearance of paralysis symptom in ALS mice was significantly latter than that in mice treated with Rilutek.

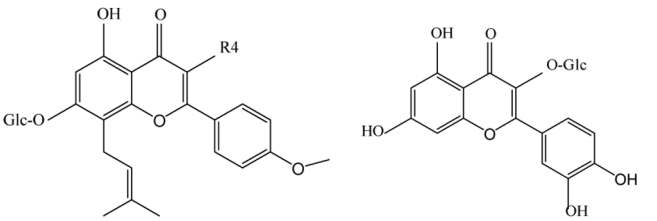
The two Chinese medicines, *Panax ginseng* and *Epimedium*, were extracted by ethanol, separated and purified by macroporous resin; finally, the extracts were made into the JWL. Presently, the chemical constituents of JWL are still not well understood, and there are no reports related to that. It is well known that chemical constituents are responsible for the therapeutic effects of drugs, meanwhile, it is believed in TCM theory that interaction of multiple chemical compounds contributes to the therapeutic effects of Chinese medicine. Therefore, the identification and analysis of multiple components are considered to be necessary and helpful for the quality evaluation of Chinese medicine.

Up to now, various analytical techniques, such as TLC, HPLC, HPLC-MS, and CZE, have been reported for the analysis of *Panax ginseng*[13–16] or *Epimedium*.[17–20] However, few methods have been reported for the simultaneous identification and quantitative analysis of the *Panax ginseng* and *Epimedium*. Generally, the constituents in JWL are complex, and some of them usually are of low content. It is particularly difficult to simultaneously isolate and determine much more active constituents by conventional HPLC-UV or HPLC-ESLD method in a short analysis time. Consequently, there is a great need for a rapid and sensitive method



20(R)-ginsenoside Rg_3

Analytes	R1	R2	R3
Rb_1	-O-Glc^2-Glc	-H	-Glc^6-Glc
Rb_2	-O-Glc^2-Glc	-H	-O-Glc^6-Ara
Rc	-O-Glc^2-Glc	-H	-O-Glc^6-Ara(f)
Rd	-O-Glc^2-Glc	-H	-O-Glc
Re	-OH	-O-Glc^2-Rha	-O-Glc
Rf	-OH	-O-Glc^2-Glc	-OH
Rg_1	-OH	-O-Glc	-O-Glc
Rg_2	-OH	-O-Glc^2-Rha	-OH
20(S)-Rg_3	-O-Glc^2-Glc	-H	-OH



Hyperin

Analytes	R4
Icariin	-O-Rha
Epimedin A	-O-Rha^2-Glc
Epimedin B	-O-Rha^2-Xyl
Epimedin C	-O-Rha^2-Rha

FIGURE 1 Chemical structures of the analytes Glc-glucose; Rha-rhamnose; Xyl-xylose; Ara-arabinose; and Ara(f)-arabinose(furanose).

to analyze multiple components simultaneously. Liquid chromatography coupled with high sensitivity mass spectrometry (LC–MS/MS) could facilitate useful information in multi-component analysis of TCM, especially in trace ingredients which are difficult to detect by conventional analysis means.[21] In addition, electro-spray ionization mass spectrometry (ESI-MS) is a soft ionization technique that forms mainly molecular ion peaks, and the fragment ions from multi-stage tandem mass spectrometry can provide rich structural information of the compounds, which is widely used in qualitative and quantitative analysis of the compounds.[22]

In this study, a simple, rapid, and highly sensitive HPLC-MS/MS method was first established for simultaneous determination of 15 constituents in JWL, including five flavonoids (icariin, epimedin A, B, C, and hyperin) and ten ginsenosides (ginsenoside Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, 20(R)-Rg_3, 20(S)-Rg_3). The structures of 15 compounds were shown in Figure 1. During the method development, multiple-reaction monitoring (MRM) method was employed in the negative ion mode using a 3200 QTRAP® system equipped with an electrospray ionization interface. Moreover, the information dependent acquisition (IDA) method was used to trigger enhance product ion (EPI) scans, so that 15 components had been unambiguously identified. Then, 12 batches of JWL were analyzed using the developed method.

EXPERIMENTAL

Chemicals and Materials

HPLC grade acetonitrile and formic acid for liquid chromatography were purchased from Fisher Chemicals (USA.) and Diamond Technology Corporation (USA.), respectively. Analytical grade methanol used for sample preparation was purchased from Tianjin Kermel Chemical Corporation (China), Deionized water was prepared using a Heal Force PWVF Purification Water System (Shanghai Canrex Analyses Instrument Corporation Limited, China).

The reference standards of ginsenoside Rb_1 (110704-200420), ginsenoside Rb_2 (111715-200802), ginsenoside Re (110754-200822), ginsenoside Rg_1 (110703-200424), ginsenoside Rf (111719-200703), 20(R)-ginsenoside Rg_3 (110804-200301), icariin (110737-200414), and hyperin (1521-200202) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); ginsenoside Rc, Rd, Rg_2, 20(S)-ginsenoside Rg_3, and epimedin A, B, C were purchased from Chengdu Mansite Biological Co. Ltd. (China).

Twelve batches of JWL were collected from Shijiazhuang Yiling Pharmaceutical Co. Ltd. (China).

Preparation of Standard Solutions

Accurately weighed solid portions of standards were dissolved in methanol to prepare standard solutions as the stock solution: 61.7 $\mu g \cdot mL^{-1}$ for ginsenoside Rb_1, 53.4 $\mu g \cdot mL^{-1}$ for ginsenoside Rb_2, 59.7 $\mu g \cdot mL^{-1}$ for ginsenoside Rc, 53.4 $\mu g \cdot mL^{-1}$ for ginsenoside Rd, 56.4 $\mu g \cdot mL^{-1}$ for ginsenoside Re, 53.3 $\mu g \cdot mL^{-1}$ for ginsenoside Rf, 56.6 $\mu g \cdot mL^{-1}$ for ginsenoside Rg_1, 50.1 $\mu g \cdot mL^{-1}$ for ginsenoside Rg_2, 35.2 $\mu g \cdot mL^{-1}$ for 20(S)-ginsenoside Rg_3, 25.0 $\mu g \cdot mL^{-1}$ for 20(R)-ginsenoside Rg_3, 56.8 $\mu g \cdot mL^{-1}$ for icariin, 53.9 $\mu g \cdot mL^{-1}$ for epimedin A, 50.5 $\mu g \cdot mL^{-1}$ for epimedin B, 50.7 $\mu g \cdot mL^{-1}$ for epimedin C, 25.0 $\mu g \cdot mL^{-1}$ for hyperin. The stock solution was diluted step by step with 50% methanol to obtain working standard solutions at five concentration levels to make calibration curve. All solutions were stored in the refrigerator at 4°C for analysis.

Sample Preparation

100 mg of JWL was accurately weighed into a 25 mL volumetric flask, dissolved in 50% methanol, and made up to volume. All the samples were diluted 10 times using 50% methanol and filtered through 0.22 μm membrane filters before analysis.

HPLC-MS Analysis

HPLC condition: The analytical column was an Agilent ZORBAX Eclipse XDB-C_{18} HPLC column (4.6 × 150 mm, 5 μm) and the oven temperature was maintained at 25°C. 0.1% formic acid aqueous solution (*v*/*v*, solvent A) and acetonitrile (solvent B) were used as mobile phase for the HPLC separation with a flow rate of 0.7 $mL \cdot min^{-1}$, the initial composition of the mobile phase was 84:16 (A:B), the gradient elution conditions: 0–6 min, 16–38% B; 6–15 min, 38–50% B; 15–18 min, 50–95% B; 18–20 min, 95–16% B; and followed by re-equilibration to the initial conditions over 6 min.

Mass spectrometric condition: The HPLC (Agilent 1200, USA) instrument was coupled with a 3200 QTRAP™ system (AB Sciex Instruments, USA), which is a hybrid triple quadrupole-linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface. The ESI source was performed in negative ionization mode. The ESI source spray voltage was adjusted to −4500 V and the atomizing temperature was set at 650°C. Nitrogen was used in all cases: the atomization gas (gas 1), auxiliary gas (gas 2) and the curtain gas were set at 60, 65, and 25 psi, respectively. Quantification was performed using multiple-reaction monitoring (MRM) mode, with a dwell time of 40 msec for each transition.

TABLE 1 The Mass Parameters for 15 Analytes

Analytes	Chemical Formula	Selected Ion	MS1	MS2	DP (V)	CE (eV)
ginsenoside Rb_1	$C_{54}H_{92}O_{23}$	$[M\text{-}H]^-$	1107.7	88.9	−95	−100
ginsenoside Rb_2	$C_{53}H_{90}O_{22}$	$[M\text{-}H]^-$	1077.5	88.9	−80	−105
ginsenoside Rc	$C_{53}H_{90}O_{22}$	$[M\text{-}H]^-$				
ginsenoside Rd	$C_{48}H_{82}O_{18}$	$[M\text{-}H]^-$	945.6	88.9	−110	−85
ginsenoside Re	$C_{48}H_{82}O_{18}$	$[M\text{-}H]^-$				
ginsenoside Rf	$C_{42}H_{72}O_{14}$	$[M\text{-}H]^-$	799.5	59.0	−105	−110
ginsenoside Rg_1	$C_{42}H_{72}O_{14}$	$[M\text{-}H]^-$				
ginsenoside Rg_2	$C_{42}H_{73}O_{13}$	$[M\text{-}H]^-$	783.5	58.9	−100	−100
20(S)-ginsenoside Rg_3	$C_{42}H_{73}O_{13}$	$[M\text{-}H]^-$				
20(R)-ginsenoside Rg_3	$C_{42}H_{73}O_{13}$	$[M\text{-}H]^-$				
icariin	$C_{33}H_{40}O_{15}$	$[M+HCOOH\text{-}H]^-$	721.2	513.1	−26	−27
epimedin A	$C_{39}H_{50}O_{20}$	$[M\text{-}H]^-$	837.2	675.2	−35	−23
epimedin B	$C_{38}H_{48}O_{19}$	$[M\text{-}H]^-$	807.3	645.2	−40	−24
epimedin C	$C_{39}H_{50}O_{19}$	$[M\text{-}H]^-$	821.3	659.3	−40	−25
hyperin	$C_{21}H_{20}O_{12}$	$[M\text{-}H]^-$	463.1	300.0	−66	−35

Collision cell exit potential (CXP) and entrance potential (EP) was set at −5.0 V and −10.0 V, respectively. The Mass parameters for 15 analytes were listed in Table 1. Instrument control and data acquisition were carried out with Applied Biosystems/MSD Sciex Analyst software (version 1.4.2).

Method Validation

Specificity

After the optimum conditions had been established, the method validation was performed. A solution containing a mixture of the freeze-dried power excipients was prepared using the JWL sample preparation procedure and injected into HPLC-MS system to evaluate possible interfering peaks.

Linearity of Calibration Curves, Limits of Detection, and Limits of Quantification

The linearity of calibration curves were made up by five chemical standards with different concentrations. Each concentration was analyzed in triplicate. The volume of standard solution injected into the HPLC-MS system was 20 μL. The limits of detection (LOD) under the chromatographic conditions were determined at the signal-to-noise ratio of 3 as criteria, while the limits of quantification (LOQ) in this study were determined as the lowest concentration point of calibration curve.

Precision

Intra-day and inter-day variations were chosen to determine the precision of the developed method. The intra-day variability was evaluated

by the six replicates analyses of the JWL samples within one day. The inter-day variability was examined by the replicate analyses of the JWL samples in three consecutive days.

Accuracy

The recovery study was used to evaluate the accuracy of the method. Three different concentration levels (approximately equivalent to 0.8, 1.0, and 1.2 times of the concentration of the sample) of the reference standards were added into a certain amount of JWL sample (0.05 g) which had been determined previously. The mixture solutions were extracted and analyzed using the method mentioned previously. The experiments were repeated three times at each level. The recovery was calculated according to the formula: Recovery (%) = 100% × (found amount – original amount)/added amount.

Matrix Effect

Evaluating matrix effect is a major problem when developing a LC–MS/MS method. Standard addition is an effective method for evaluating the matrix effect.[23] In this study, the JWL sample was extracted as described in sample preparation, then 1 mL of the extract was spiked with a one-fold mixed standard solution at three concentration levels (low, middle, and high), and another 1 mL of the extract was diluted one-fold with 50% methanol. The mixtures were vortex-mixed for 60 s, and then filtered through 0.22 μm microporous membrane. Triplicate samples were prepared at each level and each sample was analyzed three times. The matrix effect was calculated via the following formula: Matrix effect (%) = (A − B)/C × 100%, where A and B are the peak area of the analyte in the spiked sample matrix and in the diluted sample matrix, respectively. C is the peak area of the standard solutions in 50% methanol at equivalent concentrations. Generally, no matrix effect is observed when matrix effect (%) is equal to 100%, while values over 100% or lower than 100% indicate ionization enhancement or ionization suppression.[24]

RESULTS AND DISCUSSION

Optimum Conditions for HPLC–MS Analysis

All factors related to MS performance including ionization mode, declustering potential, collision energy and mobile phase have been experimented. The mass spectral conditions were optimized in both positive and negative ion modes, the negative ion mode was found to be more sensitive than positive mode in LC-MS system. The precursor ion was isolated and

dissociated into product ions with electrospray ionization interface, several fragment ion peaks of the investigated compounds were observed in mass spectra and the higher response intensities and the most stability fragment ions were chosen for quantification. Multiple-reaction monitoring (MRM) mode was used for quantitative analysis and the intensity of the molecular ion peak was further considered. The declustering potential (DP) and the collision energy (CE) are the most significant mass spectrometer parameters impacting ion response, DP and CE of each analyte were optimized to obtain the maximum sensitivity of product ions. The most suitable DP and CE were selected by observing the maximum response in MRM mode.

Ginseng saponins with the same nucleus may be dissociated into the same fragment ions in MS, which may resulted in the compounds interfere with each other and not accurate quantification if only on the basis of ion-pair. Among the 15 analysts, four pairs of ginsenoside isomers (ginsenoside Rb_2 and Rc, ginsenoside Rd and Re, ginsenoside Rf and Rg_1 as well as ginsenoside Rg_2, 20(S)-Rg_3 and 20(R)-Rg_3) were differentiated and determined through adjusting the composition and proportion of the mobile phase.

The pH of the mobile phase played an important role in the analysis of 15 components. A certain amount of formic acid was added into the mobile phase to increase signal response and improve peak shape, 0.01%, 0.05%, and 0.1% formic acid aqueous solution were compared, the results indicated that 0.1% formic acid aqueous solution and acetonitrile as the mobile phase could improve peak shape and the ionization efficiency under the ESI^- mode. It is worthwhile to mention that the initial acetonitrile content in the mobile phase was important to generate a sharp peak. Higher initial percentage of acetonitrile (>40%) generated peak splitting, whereas, a lower percentage of that (<15%) caused a long run time. Therefore, the conditions of initial proportion, gradient program and flow rate of the mobile phase were examined and optimized, respectively. The results demonstrated that 15 components could be baseline separated and eluted within 20 min when the presence of gradient program and flow rate of the mobile phase were selected.

Identification of 15 Compounds

In this study, LC-ESI-MS/MS method was applied to verify the peaks found both in standards and samples. The characteristic fragmentation patterns of the compounds were investigated using $ESI\text{-}MS^2$ in negative ion mode; the information dependent acquisition (IDA) method was employed to trigger the enhanced product ion (EPI) scans by MRM signals. All peaks of the target compounds were unequivocally identified by comparing the

TABLE 2 The Retention Time, M.W., and MS Fragmentation Ions of the Analytes

Analytes	T_R (min)	M.W.	MS (m/z)
ginsenoside Rb_1	9.28	1109	1107.7[M−H]$^-$, 945.8[M−H−Glc]$^-$, 783.5[M−H−2Glc]$^-$, 621.7[M−H−3Glc]$^-$, 459.5[M−H−4Glc]$^-$
ginsenoside Rb_2	9.84	1079	1077.5[M−H]$^-$, 945.6[M−H−Ara]$^-$, 915.1[M−H−Glc]$^-$, 783.7[M−H−Ara-Glc]$^-$, 621.4[M−H−Ara-2Glc]$^-$, 459.5[M−H−Ara-3Glc]$^-$
ginsenoside Rc	9.59	1079	1077.5[M−H]$^-$, 945.5[M−H−Ara(f)]$^-$, 915.6[M−H−Glc]$^-$, 783.4[M−H−Ara-Glc]$^-$, 621.2[M−H−Ara(f)-2Glc]$^-$, 459.2[M−H−Ara(f)-3Glc]$^-$
ginsenoside Rd	10.64	947	945.6[M−H]$^-$,783.7[M−H−Glc]$^-$,621.5[M−H−2Glc]$^-$, 459.6[M−H−3Glc]$^-$
ginsenoside Re	7.41	947	945.5[M−H]$^-$, 799.5[M−H−Rha]$^-$, 783.7[M−H−Glc]$^-$, 637.4[M−H−Glc-Rha]$^-$, 475.4[M−H−2Glc-Rha]$^-$
ginsenoside Rf	9.61	801	799.5[M−H]$^-$, 637.2[M−H−Glc]$^-$, 475.3[M−H−Glc]$^-$
ginsenoside Rg_1	7.43	801	799.5[M−H]$^-$, 637.2[M−H−Glc]$^-$, 475.2[M−H−Glc]$^-$
ginsenoside Rg_2	10.36	785	783.5[M−H]$^-$, 637.3[M−H−Rha]$^-$, 475.4[M−H−Rha-Glc]$^-$
20(S)-ginsenoside Rg_3	17.29	785	783.5[M−H]$^-$, 621.6[M−H−Glc]$^-$, 459.5[M−H−2Glc]$^-$
20(R)-ginsenoside Rg_3	17.71	785	783.5[M−H]$^-$, 621.4[M−H−Glc]$^-$, 459.4[M−H−2Glc]$^-$
Icariin	8.99	677	721.2[M+HCOO]$^-$, 529.2[M−HCOOH-Rha]$^-$, 513.1[M−HCOOH-Glc]$^-$, 367.0[M−HCOOH-Rha-Glc]$^-$
epimedin A	8.29	839	837.2[M−H]$^-$, 675.2[M−H−Glc]$^-$, 529.3[M−H−Glc-Rha]$^-$, 513.3[M−H−2Glc]$^-$, 365.9[M−H−2Glc-Rha]$^-$
epimedin B	8.44	809	807.3[M−H]$^-$, 645.2[M−H−Glc]$^-$, 366.2 [M−H−Glc-Rha-Xyl]$^-$
epimedin C	8.59	823	821.3[M−H]$^-$, 659.3[M−H−Glc]$^-$, 367.6[M−H−Glc-2Rha]$^-$
hyperin	6.78	464	463.1[M−H]$^-$, 300.0[M−H−Glc]$^-$, 255.0[M−H−Glc-HCOOH]$^-$

mass spectra of the compounds and retention time with the reference standards in MRM-IDA-EPI spectra, the dissociation rules of precursor ion were consistent with the literature.[13,25,26] The retention time, M.W. and MS fragmentation of the analytes were summarized in Table 2.

Method Validation

The chromatogram obtained from the mixture of the freeze-dried power excipients demonstrated no interfering peaks in the same retention time of standard solutions and JWL solutions. Extract ions chromatograms (XICs) of the freeze-dried power excipients, standards, and JWL recorded by HPLC-MS/MS were shown in Figure 2.

The linearity of calibration curves for the analytes were generated by plotting the peak area and the concentration using a least square regression analysis. The calibration curves were obtained using five calibration standard mixture solutions over the range of 2.50–1234 $ng \cdot mL^{-1}$. The linear correlation coefficients (r^2) for all calibration curves were greater than

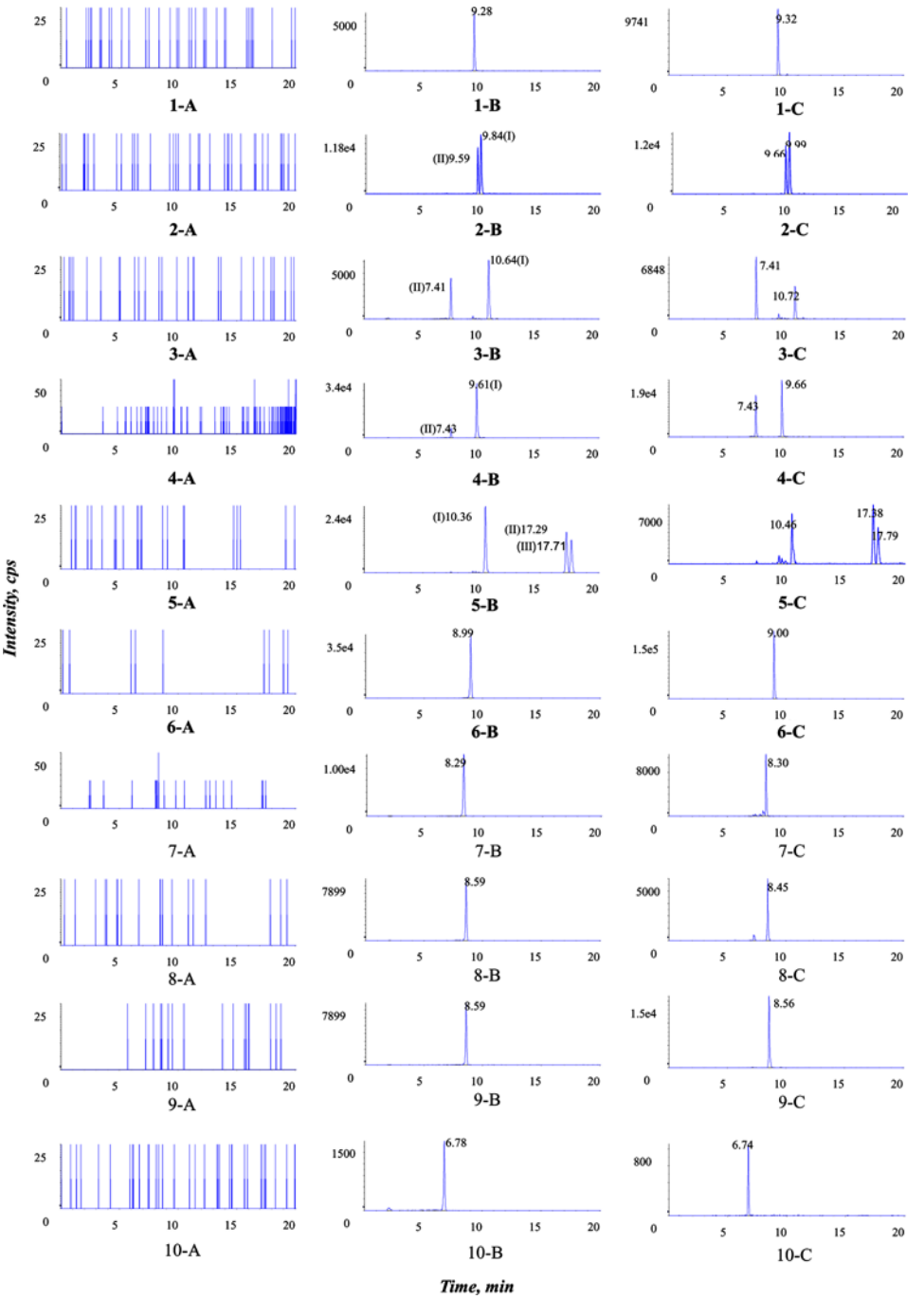


FIGURE 2 Extracted ion chromatograms of the fifteen components A: Freeze-dried power excipients, B: Standard, C: JWL. 1-Ginsenoside Rb_1; 2-Ginsenoside Rb_2(I) and Rc(II); 3-Ginsenoside Rd(I) and Re(II); 4-Ginsenoside Rf(I) and Rg_1(II); 5-Ginsenoside Rg_2(I), 20(S)-Rg_3(II), 20(R)-Rg_3(III); 6-Icariin; 7-Epimedin A; 8-Epimedin B; 9-Epimedin C; and 10-Hyperin. (Figure available in color online.)

0.998. The LOD and LOQ for the fifteen components in this study ranged from 0.25 to 1.23 $ng \cdot mL^{-1}$ and 2.50 to 6.17 $ng \cdot mL^{-1}$, respectively. These LOQ values were sufficient for the analysis of precision and accuracy. The relative standard deviations (RSD) for intra- and inter-day variations were both less than 4.52% for all analytes. These results showed that the developed method was reproducible with good precision. Related data obtained from these experiments were described in Table 3.

The overall recoveries of the 15 investigated compounds were within the range of 96.11–101.24% with RSD ranging from 1.20 to 4.10%. These tests indicated that the developed method was a reliable and available method for assessment of the quality of JWL. All data were collected and depicted in Table 4.

In the present study, standard addition was used to evaluate the matrix effect. The matrix effect data at three concentrations are listed in Table 5, showing no significant matrix effect was observed.

Sample Analysis

The samples were initially analyzed by HPLC-UV using two different detecting wavelengths, namely, 203 nm for ginsenosides and 270 nm for flavonoid glycosides. A gradient program of 75 min was used to obtain the baseline separation of the complex constituents of these samples, which result in time-consuming and solvent-wasting. Furthermore, the response of ginsenosides were lower for lacking UV-absorbing chromophore in UV spectra, ginsenoside Rg_2 and Rg_3 with lower contents cannot be successfully detected. Then, HPLC-ELSD was employed to analysis these samples, the results are similar with that of HPLC-UV method. The identification of chemical compounds is helpful to explain the pharmacological effects of Chinese medicine; thus, it is necessary to detect the trace components in JWL.

The validated analytical method with high sensitivity was subsequently applied to simultaneous determination 15 bioactive ingredients in 12 batches of JWL. Each sample was analyzed three times and the mean contents were tabulated in Table 6. Among the quantified compounds, the content of icariin is the highest, followed by ginsenoside Rb_1, Rc, Rb_2, Rg_1, and epimedin C; the four trace constitutes (Hyperin, ginsenoside Rg_2, 20(R)-ginsenoside Rg_3, and 20(S)-ginsenoside Rg_3) make up about 5% of the investigated compounds.

It is well known that the quality of TCM was affected by plant origins, sources, cultivated year, harvest time, geographical climate, and environment. In this medicine, the origins and sources of the two Chinese medicines were all limited definitely. In Chinese Pharmacopeia,[27] *Radix*

TABLE 3 Calibration Curve, the Limits of Detection, the Limits of Quantification, and Precision for the Analytes

Analytes	Regression Equation	Concentration Range ($ng \cdot mL^{-1}$)	Correlation Coefficient (r^2)	LOD ($ng \cdot mL^{-1}$)	LOQ ($ng \cdot mL^{-1}$)	Precision RSD (%)	
						Intra-day (n = 6)	Inter-day (n = 3)
ginsenoside Rb_1	$Y = 106X + 92$	6.17–1234	0.9986	1.23	6.17	1.57	2.17
ginsenoside Rb_2	$Y = 204X - 391$	5.34–1068	0.9984	1.07	5.34	2.92	3.35
ginsenoside Rc	$Y = 158X + 124$	5.97–1194	0.9984	1.19	5.97	1.21	1.20
ginsenoside Rd	$Y = 188X + 31.2$	5.34–534	0.9994	1.07	5.34	2.86	2.88
ginsenoside Re	$Y = 96.2X + 178$	5.64–1128	0.9988	1.13	5.64	1.13	2.94
ginsenoside Rf	$Y = 1.02e^3X - 111$	5.33–533	0.9992	1.07	5.33	3.02	2.77
ginsenoside Rg_1	$Y = 148X + 756$	5.66–1132	0.9996	1.13	5.66	1.83	2.24
ginsenoside Rg_2	$Y = 1.04e^3X - 198$	5.01–1002	0.9990	1.00	5.01	2.16	3.14
20(S)-ginsenoside Rg_3	$Y = 1.17e^3X + 185$	3.52–704	0.9994	0.70	3.52	3.38	3.23
20(R)-ginsenoside Rg_3	$Y = 1.41e^3X + 154$	2.50–250	0.9992	0.50	2.50	3.65	3.75
icariin	$Y = 994X + 2.5e^2$	5.68–1136	0.9988	0.57	5.68	1.25	1.05
epimedin A	$Y = 395X - 35.1$	5.39–539	0.9992	1.08	5.39	2.54	3.19
epimedin B	$Y = 251X + 301$	5.52–1104	0.9990	1.10	5.52	2.97	3.27
epimedin C	$Y = 265X + 143$	5.07–1014	0.9990	1.01	5.07	2.04	2.48
hyperin	$Y = 844X + 77.8$	2.50–250	0.9990	0.50	2.50	4.52	4.11

TABLE 4 Statistical Results of Recoveries of the 15 Analytes (n = 3)

Analytes	Original Amount (mg)	Added Amount (mg)	Found Amount (mg)	Recovery (%)	RSD (%)
ginsenoside Rb_1	0.4654	0.37680	0.83800	98.82	2.16
		0.47100	0.92938	98.78	1.69
		0.56520	1.01607	97.22	1.56
ginsenoside Rb_2	0.2247	0.18560	0.40770	98.55	1.95
		0.23200	0.45693	100.38	1.20
		0.27840	0.49373	96.44	1.56
ginsenoside Rc	0.3084	0.24720	0.55330	99.03	1.32
		0.30900	0.61990	101.11	1.60
		0.37080	0.67697	99.21	1.45
ginsenoside Rd	0.0993	0.08160	0.17937	98.03	1.79
		0.10200	0.20133	100.28	2.52
		0.12240	0.21870	97.33	2.00
ginsenoside Re	0.1740	0.14240	0.31270	97.33	2.48
		0.17800	0.34700	97.46	1.31
		0.21360	0.39017	100.99	3.79
ginsenoside Rf	0.0863	0.06800	0.15433	100.03	4.10
		0.08500	0.16900	97.62	3.00
		0.10200	0.18670	98.25	3.23
ginsenoside Rg_1	0.2412	0.18800	0.42590	98.17	2.75
		0.23500	0.46993	97.61	1.17
		0.28200	0.52120	99.07	3.46
ginsenoside Rg_2	0.0509	0.04128	0.09131	97.80	3.24
		0.05160	0.10240	100.06	3.59
		0.06190	0.11110	97.00	1.60
20(S)-ginsenoside Rg_3	0.0551	0.04400	0.09873	99.01	3.66
		0.05500	0.11067	101.24	1.61
		0.06600	0.12043	98.72	2.14
20(R)-ginsenoside Rg_3	0.0321	0.02568	0.05743	98.65	3.41
		0.03210	0.06380	99.08	3.39
		0.03852	0.06918	96.11	1.47
Icariin	0.5597	0.45120	1.01110	99.98	3.39
		0.56400	1.11833	99.33	2.31
		0.67680	1.21837	97.11	1.82
epimedin A	0.0617	0.05200	0.11293	98.54	2.36
		0.06500	0.12533	98.22	3.16
		0.07800	0.13783	97.46	1.28
epimedin B	0.0568	0.04424	0.10064	98.96	2.80
		0.05530	0.11110	98.41	2.50
		0.06636	0.12153	97.28	1.46
epimedin C	0.2173	0.16960	0.38357	98.01	2.62
		0.21200	0.42883	100.10	2.40
		0.25440	0.46807	98.39	1.94
Hyperin	0.0037	0.00304	0.00669	97.56	2.15
		0.00380	0.00747	98.81	3.64
		0.00456	0.00825	99.01	3.45

Ginseng was derived from the root of *Panax ginseng*. *Herba Epimedii* was made from the dried aerial parts of five *Epimedium* species (Berberidaceae), including *Epimedium brevicornum Maxim.*, *Epimedium sagittatum (Sieb. et Zucc.)*

TABLE 5 Matrix Effect Data for 15 Analytes in Jiweiling Freeze-Dried Powder (n = 3)

Analytes	Concentration ($ng \cdot mL^{-1}$)	Matrix Effect (%)	RSD (%)
ginsenoside Rb_1	942.0	98.10	4.81
	1884.0	95.93	2.55
	3768.0	100.81	2.07
ginsenoside Rb_2	464.0	97.81	5.73
	928.0	92.82	2.04
	1856.0	98.24	1.57
ginsenoside Rc	618.0	100.57	3.59
	1236.0	95.83	3.22
	2472.0	100.24	2.21
ginsenoside Rd	204.0	95.38	6.62
	408.0	101.46	2.22
	816.0	97.30	1.65
ginsenoside Re	356.0	94.47	4.15
	712.0	96.81	1.92
	1424.0	99.31	4.54
ginsenoside Rf	170.0	102.24	3.18
	340.0	100.75	2.08
	680.0	99.48	1.49
ginsenoside Rg_1	470.0	96.85	4.05
	940.0	99.04	1.96
	1880.0	98.93	2.76
ginsenoside Rg_2	103.2	102.71	4.77
	206.4	94.71	2.59
	412.8	100.98	2.19
20(S)-ginsenoside Rg_3	110.0	93.23	3.20
	220.0	99.26	3.84
	440.0	98.34	0.91
20(R)-ginsenoside Rg_3	64.2	101.57	4.73
	128.4	98.63	3.15
	256.8	94.81	1.24
Icariin	1128.0	97.27	4.09
	2256.0	93.13	1.68
	4512.0	96.93	2.03
epimedin A	130.0	95.13	3.21
	260.0	98.18	3.02
	520.0	100.70	3.63
epimedin B	110.6	103.84	3.80
	221.2	96.71	3.65
	442.4	95.82	3.23
epimedin C	424.0	93.57	2.28
	848.0	97.85	1.98
	1696.0	102.08	1.71
Hyperin	7.6	94.29	3.68
	15.2	96.39	2.27
	30.4	103.30	2.39

Maxim., Epimedium pubescens Maxim., Epimedium wushanense T. S. Ying and Epimedium koreanum Nakai. Radix Ginseng collected from the Northeast of China and *Epimedium pubescens Maxim.* collected from Shannxi in China

TABLE 6 Contents of the 15 Constituents in the 12 Batches of Samples

Analytes	071001 (mg/g)	071201 (mg/g)	080401 (mg/g)	080701 (mg/g)	081201 (mg/g)	090201 (mg/g)	090202 (mg/g)	090203 (mg/g)	090301 (mg/g)	090302 (mg/g)	090601 (mg/g)	091001 (mg/g)	RSD (%)
ginsenoside Rb_1	8.7183	8.9348	9.0012	8.8320	8.6092	8.7704	8.9629	9.0881	9.3681	9.1313	8.8204	8.3975	2.88
ginsenoside Rb_2	5.1294	5.0294	5.3849	5.4920	5.5854	5.3953	5.4830	5.7094	5.4666	5.6448	5.6021	4.9849	4.43
ginsenoside Rc	6.2045	6.0034	6.2492	6.6240	6.1918	6.3022	6.0103	6.0184	6.0418	6.3397	6.3042	6.2518	2.90
ginsenoside Rd	1.9944	2.0184	2.1385	2.0084	2.2631	2.1049	2.3119	1.9203	1.9722	2.2880	2.0492	1.9270	6.67
ginsenoside Re	3.1395	3.0024	3.2506	3.5502	3.1613	3.2956	3.2854	3.5577	3.4655	2.9992	3.0042	3.0947	6.33
ginsenoside Rf	1.5830	1.4860	1.6392	1.4863	1.3621	1.3968	1.4038	1.6651	1.7151	1.3722	1.3053	1.3092	9.65
ginsenoside Rg_1	5.0362	4.7304	4.6306	4.7293	4.6971	4.6640	4.8035	4.8078	4.8093	4.8340	4.7438	4.7480	2.18
ginsenoside Rg_2	0.8494	0.8603	0.9035	0.9124	0.8925	0.8604	0.8740	0.9412	0.9851	0.8450	0.8832	0.8481	4.78
20(S)-ginsenoside Rg_3	1.0039	1.0359	1.0540	1.0384	1.0081	1.0035	1.0043	1.0581	1.0852	1.0245	1.0293	0.9052	4.35
20(R)-ginsenoside Rg_3	0.5632	0.5524	0.5822	0.5930	0.5551	0.6014	0.6173	0.5852	0.6153	0.5521	0.5924	0.5774	3.96
icariin	11.034	10.958	11.105	11.305	11.0630	11.307	10.985	11.228	11.048	10.911	11.148	11.823	2.20
epimedin A	1.2940	1.5013	1.3018	1.3845	1.3149	1.2950	1.2899	1.3057	1.2871	1.2747	1.2906	1.2590	4.98
epimedin B	1.2126	1.1149	1.0947	1.2049	1.1356	1.1103	1.1294	1.1321	1.1121	1.1207	1.1283	1.0787	3.51
epimedin C	4.5862	4.6203	4.7493	4.7102	4.5614	4.7305	4.5832	4.4941	4.4293	4.4793	4.7353	4.9252	3.05
hyperin	0.0862	0.0782	0.0756	0.0772	0.0778	0.0834	0.0746	0.0756	0.0752	0.0765	0.0784	0.0899	6.14

had been used in JWL. Additionally, the harvest time and season are also limited to obtain consistent quality of the herbs. Semi-finished products were adjusted according to the contents of each batch extract to keep the consistency in the quality of the products. RSD for the variation of each compound in 12 batches was less than 10%, which suggested that the contents of 15 ingredients in all analyzed products were similar. All data indicated that the JWL pretreatment processes and manufacturing procedure were stable and feasible.

CONCLUSION

A novel and rapid HPLC-MS/MS method was established and validated for the comprehensive analysis of JWL. This method could separate 15 bioactive constitutes in a shorter time, compared with conventional HPLC-UV or HPLC-ELSD. Furthermore, it could offer accurate information of the precursor and product ions which was helpful in the identification of the complex ingredients in JWL. The proposed method showed good precision and appropriate accuracy and was successfully used to analyze 12 batches of JWL samples. Further work should be focus on determining the rule of pharmacokinetics, pharmacodynamic tests, and clinical trials.

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