Separation and Identification of 20 Chemical Constituents in the Traditional Chinese Medicinal Preparation Shenbao Tablet by LC–ESI-MS^3

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Abstract

A sensitive and specific high-performance liquid chromatography (HPLC)–electrospray ionization multiple-stage mass spectrometry for the simultaneous separation and identification of 20 chemical constituents in the traditional Chinese medicinal preparation of the Shenbao tablet is established. The samples are separated with an Alltima C_{18} column (250 × 4.6 mm, 5 µm) by linear gradient elution using water–acetic acid (A; 100:0.5, v/v) and acetonitrile (B; 0 min, 76:24; 15 min, 70:30; 40 min, 53:47; 50 min, 30:70; and maintain 10 min) as the mobile phase at a flow rate of 1.0 mL/min. The ion trap mass spectrometer is coupled to the HPLC system. Satisfactory results are obtained within 60 min for the simultaneous separation and identification of the 20 constituents. This is the first report on the analysis of main chemical constituents in the Shenbao tablet.

Introduction

Traditional Chinese medicine (TCM) can be defined as a preparation that combines various crude drugs for the prevention and treatment of disease in accordance with a certain principle. The Shenbao tablet is a famous preparation that contains 22 medicinal herbs, including *Herba epimedii* (yinyanghuo), *Fructus psoraleae* (buguzhi), *Fructus cnidii* (shechuangzi), *Herba radix astragali* (huangqi), et al. The Shenbao tablet is used to treat impotence and spermatorrhea. It has a large proportion of the market in China and is being prepared to be exported to other countries in Asia and Europe. In order to control the quality of the preparation more efficiently, a sensitive and specific method was developed to separate and identify the main chemical constituents in the Shenbao tablet.

For the analysis of these chemical constituents in crude drugs, some high-performance liquid chromatography (HPLC) methods have been reported (1–5). However, there is no simultaneous analysis of these components in the TCM preparations. Most preparations are composed of many herbs that contain complicated chemical constituents. The study of chemical constituents of Chinese herbal preparations is more difficult and complex than that of single herbs. Therefore, there have been few reports on the simultaneous analysis of multiple constituents in preparations (6,7). In this study, the routine method of HPLC cannot meet the requirements, thus we developed a liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS–MS) method to analyze the main constituents in the Shenbao tablet.

LC–ESI-MS^n has grown into one of the most powerful analytical techniques currently available. Among its advantages, LC–ESI-MS^n has provided a high level of sensitivity and selectivity. It is widely used in the analysis of complex mixtures. By coupling LC to an ion trap mass spectrometer, structural information on an analyte can be obtained by collision-induced dissociation (CID).

This study includes MS and MS–MS analysis of some of the constituents in the Shenbao tablet. It is the first report on simultaneous separation and identification of the main chemical constituents in TCM preparation of the Shenbao tablet.

Experimental

Material and reagents

Herba epimedii (yinyanghuo), *Fructus psoraleae* (buguzhi), *Fructus cnidii* (shechuangzi), *Herba radix astragali* (huangqi), *Herba cistanches* (roucongrong), and *Radix ginseng rubra* (hongshen) were all purchased at the Tongrentang TCM shop (Nanjing, China), and acteoside (verbascoside) (I), ginsenoside Rg1 (II), ginsenoside Re (III), astragaloside (IV), psoralen (V), isopsoralen (VI), osthol (VIII), and icariin (f) were all ordered from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Epimedin A (c), epimedin B (d), epimedin C (e), and Icarisid-II (l) were all kindly contributed by the Institute of Medicinal Plant, Chinese Academy of Medical

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Sciences (Beijing, China). The Shenbao tablet was provided by the Traditional Chinese Medicinal School, China Pharmaceutical University (Nanjing, China). Alcohol and acetic acid were both analytical grade, and acetonitrile was chromatographic grade.

Apparatus

An Agilent 1100 series LC system equipped with a binary pump was connected to an Agilent G1313A autosampler. Chromatographic separation was carried out at room temperature using an Alltima C_{18} analytical column (250 × 4.6 mm, 5 µm) supplied by Alltech (Deerfield, IL). The mobile phase consisted of water–acetic acid (A; 100:0.5, v/v) and acetonitrile (B; 0 min, 76:24; 15 min, 70:30; 40 min, 53:47; 50 min, 30:70; and maintained 10 min). The flow rate was 1.0 mL/min.

Detections were performed by an Agilent 1100 diode-array detector and an Agilent 1100 ion trap mass spectrometer. For simultaneous LC–diode-array detection (DAD)–MS acquisitions, the column effluent was split using a zero-dead-volumn "T" connector, with approximately one quarter of the flow being fed to the mass spectrometer. The mass spectrometry detector (MSD) was equipped with an ESI source. The ionization mode was positive. The interface and MSD parameters were as follow: nebulizer pressure, 25 psi (N_2); dry gas, N_2 (8 L/min); dry gas temperature, 325°C; spray capillary voltage, 3500 V; skimmer voltage, 40 V; ion transfer capillary exit, 94 V; scan range, 100–1200 *m/z*; spectra average, 5; ion current control, on; target, 30,000; and dwell time, 300 ms. All data acquired were processed by Agilent Chemstation Rev. A. 09.01 software (Agilent, Palo Alto, CA).

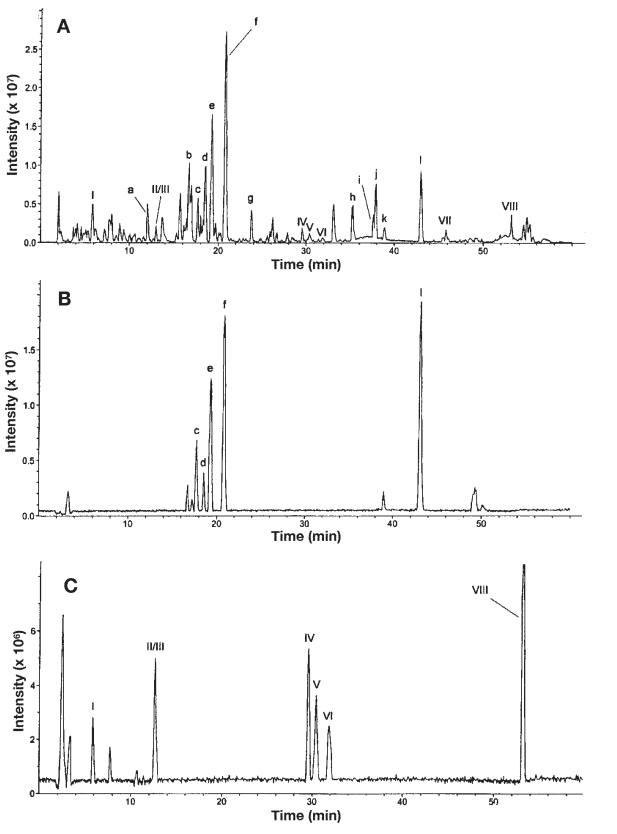


Figure 1. Full scan BPC chromatogram of the (A) Shenbao tablet and (B and C) standard compounds: epimedoside A (a), epimedin A' (b), epimedin A (c), epimedin B (d), epimedin C (e), icariin (f), unknown compound (g), sagittatoside A (h), sagittatoside B (i), 2"-*O*-rhamnosylicariside II (j), icarisid-I (k), icariside-II (l), acteoside (verbascoside) (I), ginsenoside Rg1 (II), ginsenoside Re (III), astragaloside (IV), psoralen (V), isopsoralen (VI), neobavaisoflavone (VII), and osthol (VIII).

Sample preparation

Two pieces of the Shenbao tablet (~ 1.4 g) were crushed into powder and extracted with 50 mL alcohol by refluxing on a water-bath at 100°C for 1.0 h. The extracts were diluted to 100 mL by the same solvent and filtered through a 0.45-µm filter membrane before analysis.

Results and Discussion

The Shenbao tablet is composed of 22 herbs that contain complicated chemical constituents, thus we only tried to study the main herbs of the preparation and also the major effective constituents of the main herbs. *Herba epimedii* is the most important herb in the Shenbao tablet, therefore the investigation on constituents from *Herba epimedii* is significantly important for quality control of the Shenbao tablet. We successfully separated and identified 12 main constituents (a–l) from *Herba epimedii*, and, at the same time, we identified another 8 constituents (I–VIII) (from the other 5 main herbs), which were considered the major effective constituents in the Shenbao tablet.

LC–ESI-MS–MS was employed to identify the main constituents in the Shenbao tablet. In a typical MS–MS experiment, full-scan MS spectra were first recorded during the chromatographic run, and the protonated molecules were identified. Next, product ions spectra were recorded by isolating the protonated molecule as precursor ions, followed by CID. The energy required in this process varied between 10% and 40% of the total available resonant excitation collision energy. This process was repeated to acquire MS^3 spectra that allowed not only for the identification of pseudo-molecular ions but also of specific fragments of these ions. The full-scan base peak chromatogram (BPC) is shown in Figure 1A.

Constituents from *Herba epimedii*

By comparing the mass spectral data with that of literature data (9–11), peaks c, d, e, f, and l were identified as epimedin A, B, C, icariin, and icarisid-II, respectively. Further confirmation was performed by comparing the retention times and mass spectral data with that of standard epimedin A, epimedin B, epimedin C, icariin, and icarisid-II, respectively. The main fragment ions of peaks a, b, g, h, i, j, and k were similar to that of peaks c–f, and l. They shared the same main structure and obviously originated from the same herb, *Herba epimedii*. By comparing the mass spectra with literature data (8,12,13), peaks a, b, and h–k were identified as epimedoside A, epimedin A', sagittatoside A, sagittatoside B, 2"-*O*-rhamnosylicariside II, and icarisid-I, respectively. Peak g was an unknown compound from *Herba epimedii* and will be discussed later. The structures and proposed fragmentation pathways of these constituents are shown in Figure 2. The full-scan BPC chromatogram of the standard epimedin A, epimedin B, epimedin C, icariin, and icarisid-II is shown in Figure 1B. The analysis of some of the constituents is described as follows.

Icariin

The molecular ion $[M+H]^+$ of peak f was *m/z* 677. Its MS^2 and MS^3 spectra contained fragment ions at *m/z* 531, 369, and 313. The most common decomposition processes and pathways after CID of even electrons (EE) molecular ions involve formation of new EE ions after elimination of stable, neutral molecules. The elimination of a rhamnopyranosyl moiety (146 u) produced the major fragment ions at *m/z* 531. Fragment ions at *m/z* 369 were obviously derived from the loss of a glucopyranosyl (162 u) of the $[M\text{-rham}+H]^+$ ions at *m/z* 531, and fragment ions at *m/z* 313 were derived from the loss of an isobutyl (56 u) of the ions at *m/z* 369. The proposed fragmentation pathways are shown in Figure 3. The MS, MS^2, and MS^3 spectra of icariin are shown in Figure 4A. By comparing the mass spectra data with literature data (9), peak e was identified as icariin. The main fragment ions were formed as *m/z* $677[M+H]^+$, $531[M\text{-rham}+H]^+$, $369[M\text{-rham-glc}+H]^+$, and $313[M\text{-rham-glc-isobu}+H]^+$. Further confirmation was performed by comparing the retention time and the mass spectra with that of standard icariin.

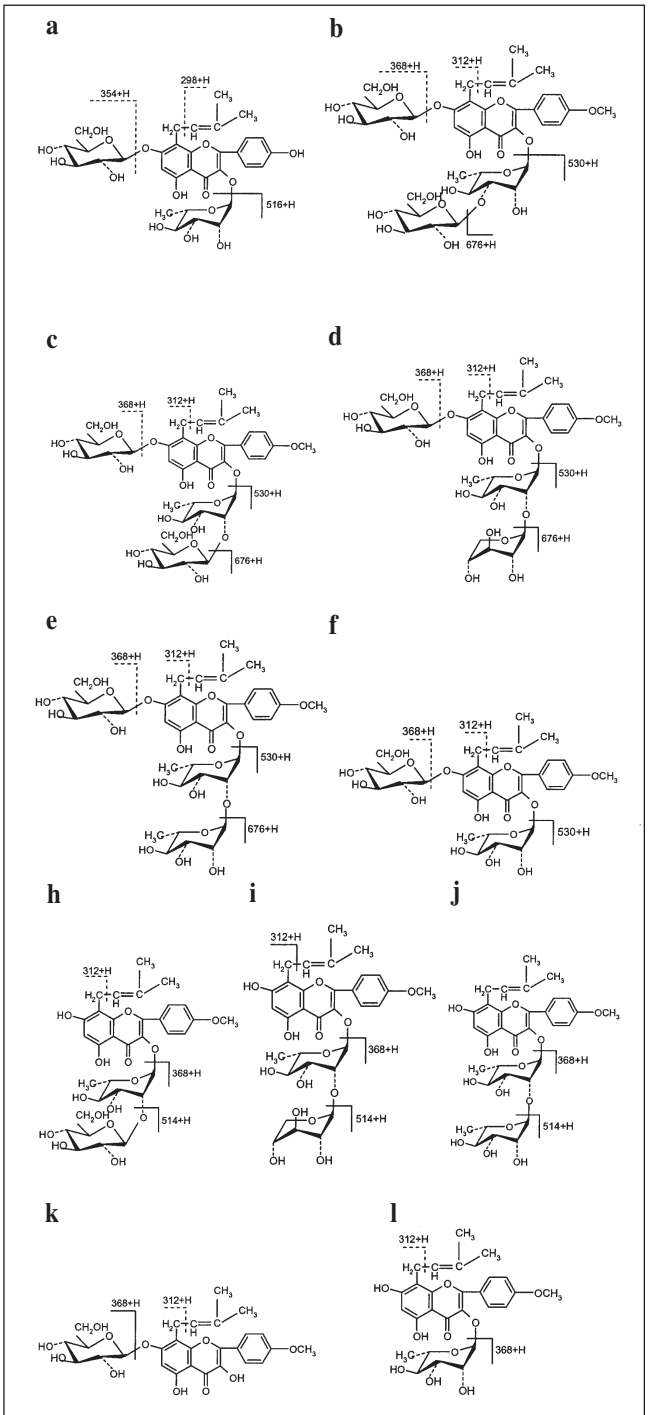


Figure 2. Proposed fragmentation pathways of constituents from *herba Epimedii*: (a) epimedoside A, *m/z* 663; (b) epimedin A', *m/z* 839; (c) epimedin A, *m/z* 839; (d) epimedin B, *m/z* 809; (e) epimedin C, *m/z* 823; (f) icariin, *m/z* 677; (h) sagittatoside A, *m/z* 677; (i) sagittatoside B, *m/z* 647; (j) 2"-*O*-rhamnosylicariside II, *m/z* 661; (k) ecarisid-I, *m/z* 531; and (l) icarisid-II, *m/z* 515. Solid line = MS^2 fragmentation and dashed line = MS^3 fragmentation.

Epimedin A, B, and C

The mass spectra of peak c showed fragment ions at *m/z* 839, 677, 531, 369, and 313. The mass spectra of peak d showed fragment ions at *m/z* 809, 677, 531, 369, and 313, and the mass spectra of peak e showed fragment ions at *m/z* 823, 677, 531, 369, and 313. They shared the same fragment ions at *m/z* 677, 531, 369, and 313 with icariin, which must have the same fragmentation pathways. For peak c, d, and e, fragment ions at *m/z* 677 were derived from the loss of a glucopyranosyl (162 u), xylopyranosyl (132 u), and rhamnopyranosyl (146 u), respectively. According to the literature data (10), peaks c, d, and e were identified as epimedin A, B, and C, respectively. The main fragment ions of peak c were formed as *m/z* $839[M+H]^+$, $677[M\text{-glc}+H]^+$, $531[M\text{-glc-rham}+H]^+$, $369[M\text{-glc-rham-glc}+H]^+$, and $313[M\text{-glc-rham-glc-isobu}+H]^+$. The main fragment ions of peak d were formed as *m/z* $809[M+H]^+$, $677[M\text{-xyl}+H]^+$, $531[M\text{-xyl-rham}+H]^+$, $369[M\text{-xyl-rham-glc}+H]^+$, and $313[M\text{-xyl-rham-glc-isobu}+H]^+$ and the main fragment ions of peak e were formed as *m/z* $823[M+H]^+$, $677[M\text{-rham}+H]^+$, $531[M\text{-rham-rham}+H]^+$, $369[M\text{-rham-rham-glc}+H]^+$, and $313[M\text{-rham-rham-glc-isobu}+H]^+$. By comparing the retention times and mass spectra with those of standard epimedin A, B, and C, peaks c, d, and e were further identified as epimedin A, B, and C, respectively.

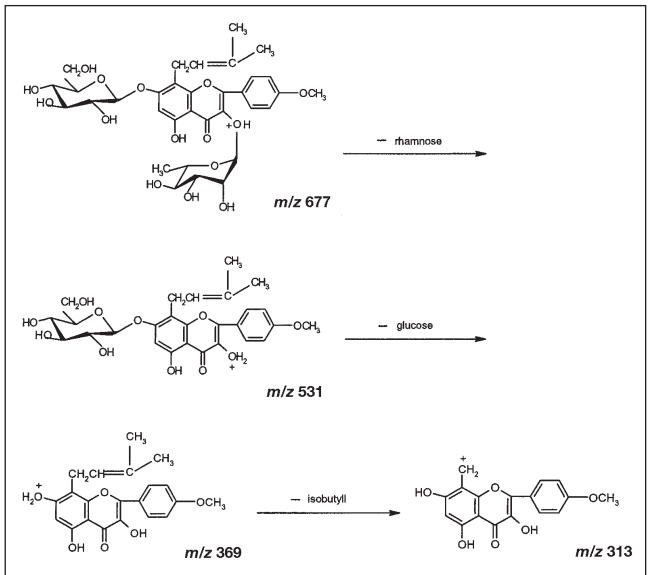


Figure 3. Proposed MS–MS fragmentation pathways of icariin.

Icarisid-II

The mass spectrum of peak l showed fragments at *m/z* 515, 369, and 313, which was similar to the MS pattern of icarisid-II (11) (reported as *m/z* 515, 369, and 313). By comparing its retention time and mass spectrum with that of standard icarisid-II, peak j was identified as icarisid-II.

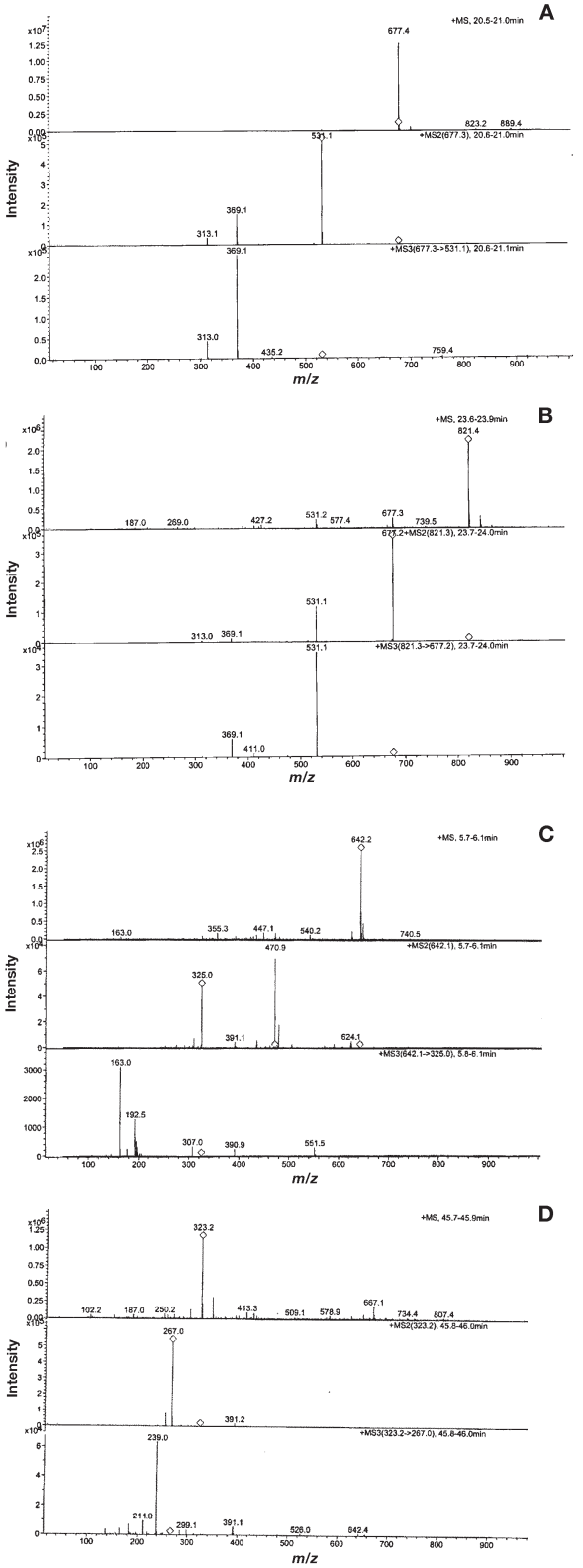


Figure 4. MS, MS^2, and MS^3 mass spectrum: (A) icariin, *m/z* 677; (B) unknown compound, *m/z* 821; (C) acteoside (verbascoside), *m/z* 642; and (D) neobavaisoflavone, *m/z* 323.

Epimedoside A

The mass spectrum of peak a showed prominent peaks at *m/z* 663, 517, 355, and 299, which was very similar to the spectrum of epimedoside A (12). Based on the mass spectral data, peak a was identified as epimedoside A. The main fragment ions were formed at *m/z* 663$[M+H]^+$, 517$[M\text{-}rham+H]^+$, 355$[M\text{-}rham\text{-}glc+H]^+$, and 299$[M\text{-}rham\text{-}glc\text{-}isobu+H]^+$.

Sagittatoside A and B

The mass spectrum of peak h showed fragment peaks at *m/z* 677, 515, 369, and 313, and peak i showed fragment peaks at *m/z* 647, 515, 369, and 313. By comparing with literature data (11), it could be observed that the mass spectra of peaks h and i were similar to sagittatoside A and sagittatoside B, respectively. The main fragment ions of peak h were formed as *m/z* 677$[M+H]^+$, 515$[M\text{-}glc+H]^+$, 369$[M\text{-}glc\text{-}rham+H]^+$, and 313$[M\text{-}glc\text{-}rham\text{-}isobu+H]^+$, and the main fragment ions of peak i were formed as *m/z* 647$[M+H]^+$, 515$[M\text{-}xyl+H]^+$, 369$[M\text{-}xyl\text{-}rham+H]^+$, and 313$[M\text{-}xyl\text{-}rham\text{-}isobu+H]^+$.

2"-O-rhamnosylicariside II

By comparing the mass spectral data of peak j with that of 2"-*O*-rhamnosylicariside II (13), peak j was identified as 2"-*O*-rhamnosylicariside II, for which the mass spectrum was reported as *m/z*

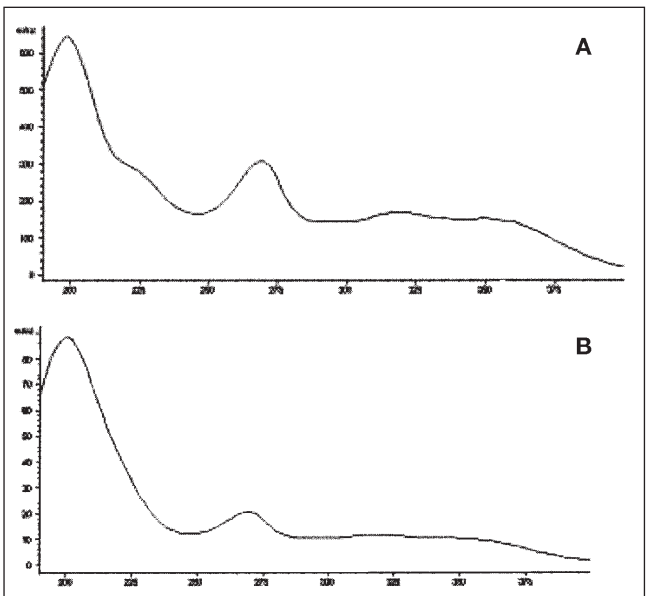


Figure 5. UV spectrum of icariin (A) and the unknown compound (B).

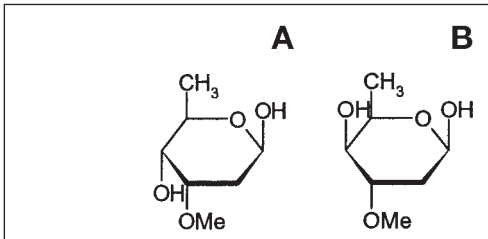


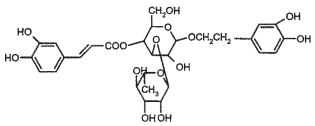
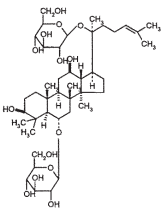
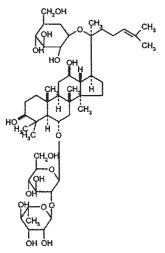
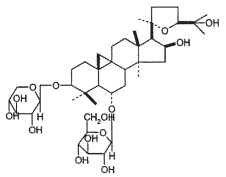
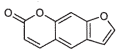
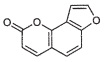
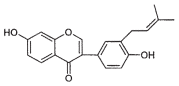
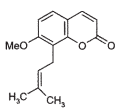
Figure 6. Structures of cymarose (A) and sarmantose (B).

661, 515, 369, and 313. The main fragment ions were formed as *m/z* 661[M+H]$^+$, 515[M-rham+H]$^+$, 369[M-rham-rham+H]$^+$, and 313[M-rham-rham-isobu+H]$^+$.

The unknown compound

Peak g was an unknown compound that no report had been found to mention. Its mass spectrum showed fragments at *m/z* 843, 821, 677, 531, 369, and 313. It shared the same fragment ions 677, 531, 369, and 313 with icariin (f), and it was clear that the unknown compound was a close relative of icariin isolated from the same source. The UV spectra of peak g were similar to that of icariin (Figure 5). The fragment ions at *m/z* 821 and *m/z* 843 were the molecular ions species [M+H]$^+$ and [M+Na]$^+$, respectively, and the fragment ions at *m/z* 677 may be derived from the loss of a sugar portion (144 u) from [M+H]$^+$. By consulting the reference book (14,15) it was found that the most possible structures of the sugar moiety were that of cymarose and sarmantose. The structures of cymarose and sarmantose are shown in Figure 6. The base peak in the MS^3 spectrum at *m/z* 531 may derive from the loss of a rhamnopyranosyl moiety (146 u) of the ions at *m/z* 677, and the fragment ions at *m/z* 369 may derive from the loss of a glucopyranosyl (162 u) of the ions at *m/z* 531. The MS, MS^2, and MS^3 spectra of peak g are shown in Figure 4B. Further research work is in process.

Table I. Names, Structures, Retention Times, UV, and Mass Spectral Data for Constituents I–VIII

Constituent	Belong to (herb)	t_R (min)	UV λ_{max}	MW	Structure	MS data
Acteoside	Herba Ciztanches (Roucongrong)	7.8	218, 285,333	624		642[M+NH$_4$]$^+$, 624[M-H$_2$O+NH$_4$]$^+$, 471[M-C$_8$H$_9$O$_3$]$^+$, 325[M-C$_8$H$_9$O$_3$-rham]$^+$, 163[M-C$_8$H$_9$O$_3$-rham-glc]$^+$
Ginsenoside Rg1	Radix Ginseng Rubra (Hongshen)	12.9	203	800		823[M+Na]$^+$, 767[M-isobu+Na]$^+$
Ginsenoside Re		12.9	203	946		969[M+Na]$^+$, 823[M-rham+Na]$^+$, 767[M-isobu+Na]$^+$
Astragaloside	Radix Astragali (Huangqi)	29.8	205	784		807[M+Na]$^+$
Psoralen	Eructus Psoraleae (Buguzhi)	30.3	205, 245, 292, 333	186		187[M+H]$^+$, 395[2M+Na]$^+$
Isopsoralen		31.7	213, 245, 299	186		187[M+H]$^+$, 395[2M+Na]$^+$
Neobavaisoflavone		45.8	–	322		667[2M+Na]$^+$, 323[M+H]$^+$, 267[M-isobu+H]$^+$, 239[M-CO-isobu+H]$^+$, 211[M-CO-CO-isobu+H]$^+$
Osthol	Fructus Cnidii (Shechuangzi)	53.1	205, 258,320	244		511[2M+Na]$^+$, 267[M+Na]$^+$, 189[M-isobu+H]$^+$

Epimedoside C and icaritin

Peaks of another two compounds from *Herba epimedii* were found in the full-scan BPC chromatogram. By comparing their mass spectra with literature data (12), the two peaks were identified as epimedoside C (t_R, 24.8 min; *m/z* 517[M+H]$^+$ and 355[M-rham+H]$^+$) and icaritin (t_R, 45.5 min; *m/z* 369[M+H]$^+$ and 313[M-isobu+H]$^+$), respectively.

The other eight main constituents

Peaks I, II, III, IV, V, VI, VII, and VIII were identified as acteoside (verbascoside), ginsenoside Rg1, ginsenoside Re, astragaloside, psoralen, isopsoralen, neobavaisoflavone, and osthol, respectively, by comparison of the molecular weight and the mass spectral data with literature data (8,16–19). Further identification was performed by comparing the retention times, UV, and mass spectra with those of the standard acteoside (verbascoside), ginsenoside Rg1, ginsenoside Re, astragaloside, psoralen, isopsoralen, and osthol, respectively. The full-scan BPC chromatogram of the standard compounds is shown in Figure 1C. The structures, retention times, and mass data are shown in Table I. The analysis of some of the constituents is described as follows.

Acteoside (verbascoside)

The mass spectrum of peak I showed fragment peaks at *m/z* 642, 624, 471, 325, and 163. The molecular weight and structure information provided by the mass spectrum of peak I was in accordance with that of acteoside (verbascoside) (8,16). By comparing the retention time and mass spectrum of peak I with that of standard acteoside (verbascoside), peak I was identified as acteoside (verbascoside). The main fragment ions were formed as *m/z* 642[M+NH$_4$]$^+$, 624[M-H$_2$O+NH$_4$]$^+$, 471[M-C$_8$H$_9$O$_3$]$^+$, 325[M-C$_8$H$_9$O$_3$-rham]$^+$, and 163[M-C$_8$H$_9$O$_3$-rham-glc]$^+$. The proposed fragmentation pathways are shown in Figure 7. The MS, MS^2, and MS^3 mass spectra of acteoside (verbascoside) are shown in Figure 4C.

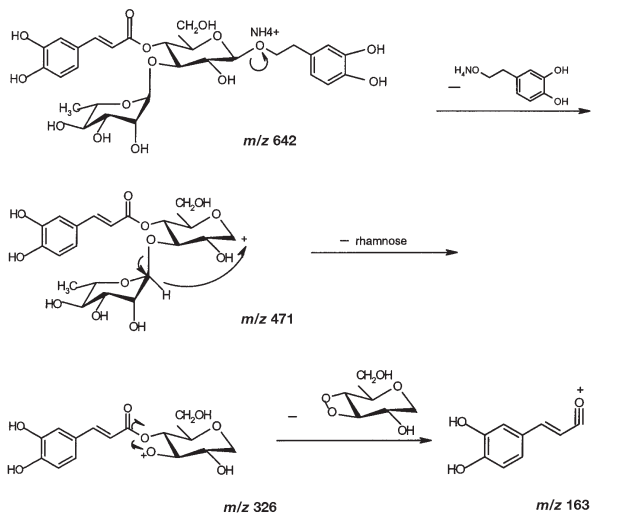


Figure 7. Proposed MS–MS fragmentation pathways of acteoside (verbascoside).

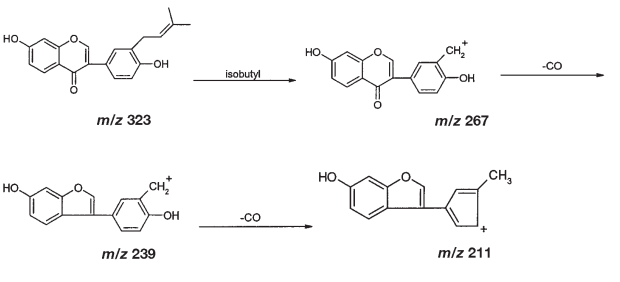


Figure 8. Proposed MS–MS fragmentation pathways of neobavaisoflavone.

Neobavaisoflavone

By comparing the mass spectral data of peak VII with that of neobavaisoflavone (19), peak VII was identified as neobavaisoflavone, for which the mass spectrum showed fragment peaks at *m/z* 667, 323, 267, 239, and 211. It was clear that [M+H]$^+$ at *m/z* 323 was formed, and extensive isobutyl loss (formation of the ions at *m/z* 267) took place. The base peak in the MS^3 spectrum at *m/z* 239 was derived from the loss of a CO of the [M-isobu+H]$^+$ ions at *m/z* 267. The peak at *m/z* 667 was obviously derived from the combination of two neobavaisoflavone and a Na$^+$. The main ions were formed as *m/z* 667[2M+Na]$^+$, 323[M+H]$^+$, 267[M-isobu+H]$^+$, 239[M-CO-isobu+H]$^+$, and 211[M-CO-CO-isobu+H]$^+$. The proposed fragmentation pathways are shown in Figure 8. The MS, MS^2, and MS^3 mass spectrum of neobavaisoflavone are shown in Figure 4D.

Conclusion

Twelve constituents from the main medicine *Herba epimedii* and eight other main constituents from the other five main herbs were simultaneously separated and identified in the Shenbao tablet by LC–ESI–MS^3 without any tedious pretreatment. It is a simple, reliable, and specific method for quality control of the TCM preparation Shenbao tablet.

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