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Short communication

Determination of breviflavone A and B in *Epimedium* herbs with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Two new types of minor flavonoids, breviflavone A and B, have been recently isolated and identified from *Epimedium brevicornu* in our previous research. Breviflavone B is a novel flavonoid with potent and specific estrogen receptor (ER) bioactivity. Its positional isomer, breviflavone A, is not ER active. Therefore, it is important to determine the two minor components, breviflavone A and B, in *Epimedium* herbs. In this report, a robust method for measurement of the two breviflavones in *Epimedium* ethanolic extracts has been developed by using liquid chromatography tandem mass spectrometry via selected-reaction monitoring (m/z 437 → m/z 367 for breviflavone A and m/z 437 → m/z 351 for breviflavone B) under negative electrospray ionization mode. This method has been successfully used to determine the two breviflavones in ethanolic herbal extracts of five major *Epimedium* species (*E. brevicornu*, *E. koreanum*, *E. pubescens*, *E. sagittatum*, and *E. wushanese*) from various sources. The contents of the two breviflavones range from 0.0181 to 0.1791% for breviflavone A and 0.0026 to 0.0252% for breviflavone B in the dried ethanolic extracts of those *Epimedium* herbal samples.

1. Introduction

The traditional Chinese medicinal plant, *Epimedium* L. (*Berberidaceae*), is a popular botanical supplement used to improve menopausal symptoms and bone health, amongst other indications in many countries [1–3]. Flavonoids have been proven to be the bioactive components in *Epimedium* herbs, and more than 60 flavonoids have been isolated from *Epimedium* herbal extracts [4,5]. *Epimedium* herbs contain major components, e.g., epimedium A, B, C, and icariin, etc., and many minor components such as apigenin, luteolin, kaempferol, quercetin and so on [3,4]. Two new types of minor flavonoids, breviflavone A and B, have been recently isolated and identified from *Epimedium brevicornu* in our previous research [2,3]. Breviflavone B has been found to be a novel flavonoid with specific estrogen receptor (ER) bioactivity. It increased estrogen-responsive human breast cancer cell proliferation at low doses, but paradoxically caused profound inhibition of growth at higher doses. Interestingly, high doses of breviflavone B resulted in degradation of ERα protein in breast cancer cells [2,3]. This suggests that breviflavone B is of value in estrogen-deficiency states and for prophylaxis of breast cancer. However, its positional isomer, breviflavone A, is not ER active. Therefore, it is important to develop a robust and sensitive method to determine the two minor breviflavones in the *Epimedium* herbs.

It is well-known that documentation of quality, safety and clinical efficacy are essential for acceptance of herbal drugs in mainstream medicine [6]. For the quality control of botanicals and herbal extracts, the consistency and stability of bioactive constituents of different batches of herbs are critical [3,6]. It is reported that *Epimedium* herbs have five major species (*E. brevicornu*, *E. koreanum*, *E. pubescens*, *E. sagittatum*, and *E. wushanese*) [7] and at least other thirteen minor species [3,4]. Our previous research showed that the breviflavone B is the most potent estrogenic component in those *Epimedium* species [2,3]. Therefore, robust and accurate determination of the minor bioactive component breviflavone B in the extracts is vital for quality control of the *Epimedium* herbal products.

Many analytical methods, including UV–vis spectrophotometry [8], thin layer chromatography (TLC) [9], high-performance liquid chromatography (HPLC) [10], micellar electrokinetic chromatography (MEKC) [11] and capillary zone electrophoresis (CZE) [12], have been reported for the determination of the flavonoids in *Epimedium* herbal samples. However, these methods suffered from low resolution or low sensitivity for minor flavonoids. Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been applied for qualitative and quantitative analyses of flavonoids in *Epimedium* herbal extracts [4,13] and in human [14] and rat plasma samples [15] after administration of *Epimedium*

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herbal products. Those reported LC–MS/MS methods provided high specificity, high resolution and sensitivity for determination of both major and minor flavonoids in *Epimedium* herbal samples.

In this report, a sensitive and robust LC–MS/MS method has been developed for determining the two new minor flavonoids of breviflavone A and B in *Epimedium* herbal ethanolic extracts. This LC–MS/MS method has been successfully used to measure the content of the two breviflavones in five major authenticated *Epimedium* herb species from various sources. It provides an excellent approach for rapid screening for bioactive breviflavone B from *Epimedium* species and herbal products.

2. Experimental

2.1. Instrumentation

HPLC was carried out on an Agilent Technologies (Waldbronn, Germany) Model 1100 liquid chromatograph system with a multiple wavelength UV detector. A Cadenza CD-C18 (150 mm × 2 mm, 3 μm, Imatakt, Japan) column was used for separation. The HPLC effluent was analyzed by an Agilent G2445A (controlled by 4.0.25 software) ion-trap mass spectrometer (Waldbronn, Germany) equipped with an electrospray ionization (ESI) source operated under negative mode. Chromatographic data were recorded and processed by using the DataAnalysis software version 2.2 (Bruker Dalton GmbH, Bremen, Germany).

2.2. Chemicals

Breviflavone A and B (99% purity) were isolated from *E. brevicornu* as previously reported [2]. Icariin, epimedin A, B and C were purchased from 3B Medical System, Inc (Libertyville, IL). Formic acid and HPLC-grade solvents acetonitrile, methanol and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Absolute ethanol and 4-hydroxylbenzophenone (99% purity) were obtained from Sigma–Aldrich (St. Louis, MO). Ultrapure water was prepared using a Milli-Q (Millipore, Bedford, MA) water purification system. Dried leaves of *Epimedium* herbs were obtained from China, Singapore, or Germany. All the specimens of *Epimedium* herbs were taxonomically identified by Dr. Guo Baolin [3] and compared with standard samples by genetic fluorescent amplified fragment length polymorphisms (AFLP) analysis [3]. Reference specimens were archived at Singapore Herbarium [2] and Institute for Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing [3].

2.3. Sample preparation

Dried *Epimedium* leaves were grounded into powder and soaked in 100% ethanol (1:10, w/v) at 37 °C for 7 days [2,3], filtered, and the supernatant dried in vacuum. The dried ethanol herbal extract were weighed and re-dissolved in DMSO for the determination. Stock solution of breviflavone A and B at 1 mg/mL were prepared in DMSO. A calibration curve was composed of six calibration standards of breviflavone A and B (0.0219, 0.0438, 0.219, 0.438, 2.19, 4.38 μg/mL) containing 4-hydroxylbenzophenone (1 μg/mL) as internal standard in DMSO. Nine sets of three quality control samples containing 0.0219, 0.438 and 2.19 μg/mL of breviflavone A and B and 1 μg/mL of internal standard were individually prepared in DMSO for method validation [16]. Twenty microliters of those standard solutions, quality controls, and the herbal extract samples (5 mg/mL) containing 1 μg/mL of internal standard were injected into the LC–MS/MS, after which the injector was washed with three post-injection washes with 750 mL/L methanol–250 mL/L H_2O.

2.4. High-performance liquid chromatography

HPLC was carried out with the Cadenza C18 column with a mobile phase that consisted of two eluents, solvent A (950 mL/L acetonitrile–50 mL/L H_2O containing 1 mL/L formic acid) and solvent B (50 mL/L acetonitrile–950 mL/L H_2O containing 1 mL/L formic acid), delivered at a flow rate of 0.25 mL/min. The initial condition of 20% solvent A was slowly increased to 25% solvent A for the first 12 min. This was followed by a fast linear gradient to 70% solvent A over the next 13 min and this condition was maintained for the final 5 min. Internal standard (IS), 4-hydroxylbenzophenone, was completely separated from the major components (epimedin A, B, C, and icariin) and the two minor breviflavones under this condition. The retention times of 4-hydroxylbenzophenone and breviflavone A and B were found to be 21.6, 26.0 and 26.5 min, respectively. In order to reduce the interference of polar impurities and some major chemicals and to improve detection sensitivity for breviflavones and internal standard, three time segments were applied in the analysis. In the first time segment, from 0 to 19 min, the inlet of the mass spectrometric detector (MSD) was automatically switched to waste (by-passing the MS) of each run to elute polar impurities and some major components. In the second time segment, from 19 to 24 min, the inlet was switched to the MS and the internal standard, 4-hydroxylbenzophenone, was eluted and detected. In the third time segment, from 24 to 30 min, the inlet was switched to the MSD and the breviflavone A and B were eluted and detected. Thereafter, the inlet was switched to waste for the next run.

2.5. Mass spectrometry

All mass spectrometric measurements were obtained using the Agilent ion trap mass spectrometer equipped with electrospray ionization (ESI) source, working in negative conditions. All of the source and instrument parameters were optimized by flow injection analysis of pure compounds of breviflavone A and B and 4-hydroxylbenzophenone. The ESI conditions were set as follows: capillary voltage 3.5 kV; end plate offset voltage −500 V; nebulizer (nitrogen) pressure 30 psi; drying gas (nitrogen) flow 9 L/min; temperature 350 °C. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas. The collision energy was set at 100%. ICC target was set at 8000. Maximum accumulation time was set at 200 ms. The number of average scans was 4. For determining internal standard by monitoring m/z 197 → m/z 169 + m/z 92 in the second time segment, fragmentation cutoff was set as 85 m/z. Mass scan range was set as from 85 m/z to 210 m/z. Fragmentation voltage was set at 1.35 V for 30 ms. Isolating width was set at 2 m/z. For determining breviflavone A and B via monitoring m/z 437 → m/z 367 for breviflavone A and m/z 437 → m/z 351 for breviflavone B in the third time segment, fragmentation cutoff was set as 150 m/z. Mass scan range was set as from 150 m/z to 450 m/z. Fragmentation voltage was set at 1.15 V for 30 ms. Isolating width was set at 2 m/z. The concentration of breviflavones was quantified using a six-point calibration curve of peak area ratio for breviflavones to internal standard against the concentration.

3. Results and discussion

3.1. Development of the LC–MS/MS method to measure breviflavone A and B

Breviflavone A and B are two weak acidic phenolic molecules [2]. This property suggests that chromatographic separation and retention of the two breviflavones can be enhanced by using acidic

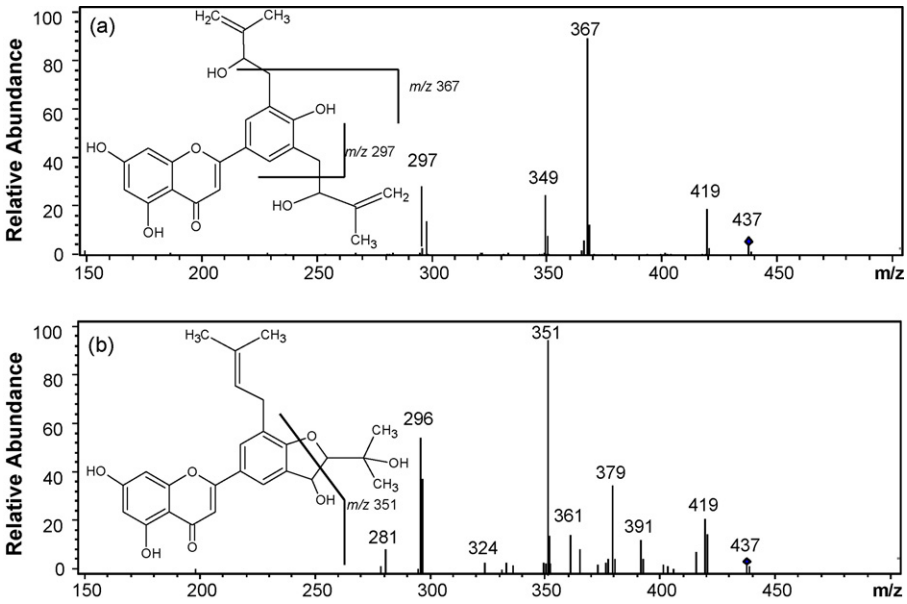

Fig. 1. Chemical structure and MS/MS fragmentation of (a) breviflavone A and (b) breviflavone B.

additive in the mobile phase [14]. The negative electrospary ionization can generally provide better ionization efficiency and could achieve better signal to noise ratio for this type of compounds in the complicated sample matrix [1,14]. We found that breviflavone A and B were exhibited excellent MS signals of $[M-H]^-$ ions at m/z 437 under negative electrospray ionization and satisfied chromatographic retention on the Cadenza C18 column by using an acetonitrile/water mixture containing 0.1% formic acid as mobile phase.

The chemical structure and MS/MS fragmentation of the breviflavone A and B are shown in Fig. 1. Breviflavone A contains two prenyl groups. A dormant daughter ion of m/z 367 was observed in the MS/MS spectrum (Fig. 1a) of breviflavone A due to loss of one prenyl group moiety. A strong peak of m/z 297 was also shown in the spectrum, corresponding to loss of two prenyl group moieties. Breviflavone B was exhibited a different MS/MS fragmentation profiling (Fig. 1b). The five member ring appeared to be easily broken and exhibited a dormant daughter ion of m/z 351 in the spectrum. We found that it is highly specific and sensitive by monitoring SRM transitions for breviflavone A and B at m/z 437 → m/z 367 and m/z 437 → m/z 351, respectively, in *Epimedium* extracts. Fig. 2 shows the typical UV chromatogram and mass chromatograms of standard solution containing mixture of 0.438 μg/mL of breviflavone A and B.

3.2. Validation of the LC–MS/MS method

The lower limits of quantization for breviflavone A and B were determined as 0.0219 μg/mL. Triplicate sets of calibration standard curves were obtained to determine the linearity of the assay. This LC–MS/MS method exhibited a good linear range from 0.0219 to 4.38 μg/mL for breviflavones. The typical equation of the calibration curves for breviflavone A and B were $Y = 0.4629X + 0.0267$ ($R^2 = 0.9996$) and $Y = 0.1177X + 0.0148$ ($R^2 = 0.9992$), respectively. To determine the interday accuracy and precision, three sets of three quality control samples [16] having concentrations of 0.0219, 0.438 and 2.19 μg/mL of breviflavone A and B were determined on each of 3 consecutive days. To determine the intraday accuracy and precision, six sets of those quality control samples were analyzed on the same day. The interday and intraday precision ranged from 5.4 to 10.0% (relative standard deviation (R.S.D.)) for breviflavone A and 4.6–12.0% for breviflavone B (shown in Table 1). Interday and intraday accuracies were 93.6–109.8% for breviflavone A and 92.2 to 107.2% for breviflavone B. In terms of accuracy and precision, all validation values fell within ±15% (Table 1). These results indicated the LC–MS/MS method was reliable for the measurement of the two minor breviflavones. We have compared both actual *Epimedium* samples and quality control samples for the method validation. No significant difference was observed for the validation results by

Table 1
Intraday and interday variations in breviflavone A and B measurements.

Breviflavone A				Breviflavone B			
Nominal concentration (μg/mL)[a]	Estimate concentration (μg/mL)[b]	Accuracy (%)	R.S.D. (%)	Nominal concentration (μg/mL)	Estimate concentration (μg/mL)	Accuracy (%)	RSD (%)
Intraday							
0.0219	0.0205	93.6	9.3	0.0219	0.0202	92.2	11.2
0.438	0.468	106.9	5.4	0.438	0.453	103.5	6.7
2.19	2.31	105.5	5.7	2.19	2.25	102.7	4.6
Interday							
0.0219	0.0211	96.3	10.0	0.0219	0.0203	92.7	12.0
0.438	0.435	99.4	8.6	0.438	0.421	96.1	10.6
2.19	2.41	109.8	5.9	2.19	2.35	107.2	6.2

[a] Three sets of triplicate quality control samples were determined on 3 consecutive days to estimate interday accuracy and precision; six sets of the three quality control samples were determined within the same day to estimate the intraday accuracy and precision.
[b] Concentration values are the mean of those tests.

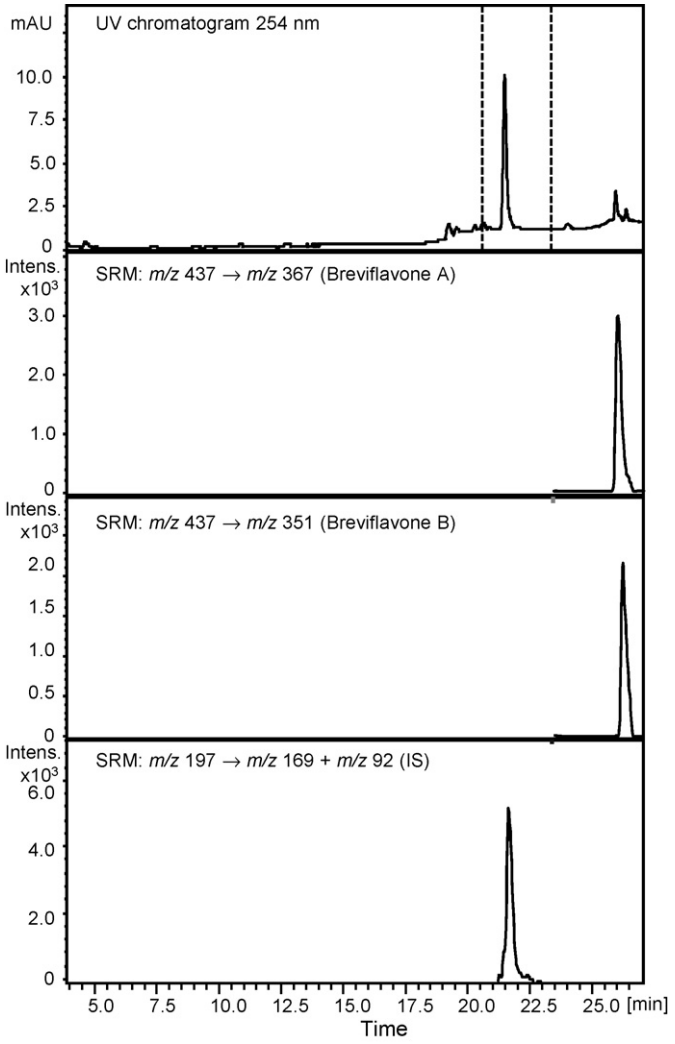

Fig. 2. Typical UV chromatogram and mass chromatograms of standard solution containing mixture of 0.438 μg/mL of breviflavone A and 0.438 μg/mL of breviflavone B. The two vertical lines in the UV chromatogram are the dividing lines of the three time segments.

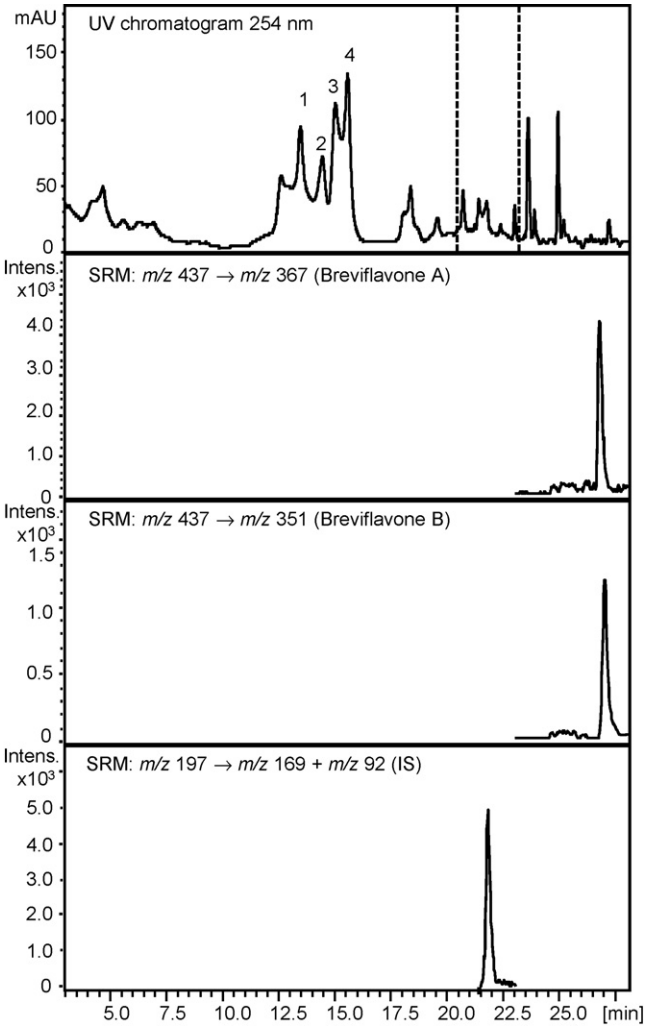

Fig. 3. Typical UV chromatogram and mass chromatograms of *Epimedium brevicornu* herbal extract. (1) Epimedin A; (2) epimedin B; (3) epimedin C; (4) icariin. The two vertical lines in the UV chromatogram are the dividing lines of the three time segments.

Table 2
Content of breviflavone A and B in five major *Epimedium* species from various sources.

Sample	Source	Breviflavone A[a]		Breviflavone B	
		Content (g/100 g)[b]	R.S.D. (%) (n = 3)	Content (g/100 g)	R.S.D. (%) (n = 3)
E. brevicornu	Singapore	0.1791	3.1	0.0121	8.2
E. brevicornu	Germany	0.0305	9.0	0.0096	5.9
E. brevicornu	Zhejiang Province, China	0.0186	2.8	0.0252	6.2
E. brevicornu	Henan Province, China	0.0386	8.7	0.0249	6.8
E. koreanum	Jilin Province, China	0.0460	8.4	0.0060	7.4
E. koreanum	Liaoning Province, China	0.1206	2.5	0.0108	7.3
E. koreanum	Germany	0.0915	3.2	0.0026	5.8
E. pubescens	Shanxi Province, China	0.0593	4.4	0.0034	7.0
E. pubescens	Sichuan Province, China	0.0191	10.9	0.0111	4.8
E. pubescens	Germany	0.1240	6.9	0.0091	4.6
E. sagittatum	Hunan Province, China	0.0511	4.0	0.0074	5.9
E. sagittatum	Germany	0.0590	6.5	0.0055	7.6
E. wushanese	Sichuan Province, China	0.0181	5.7	0.0042	7.5

[a] Contents were expressed as g/100 g of dried ethanolic extracts.
[b] Content values are the mean of three tests.

using the two type samples and no matrix effect was observed (data not shown).

3.3. Application to measure the two breviflavones in ethanolic extracts of Epimedium herbs from various sources

Epimedium extract is a complicated mixture. It contains major compounds, e.g., epimedium A, B, C and icariin, etc., and many minor compounds including breviflavone A and B. As shown in Fig. 3, the four major compounds epimedium A, B, C, and icariin were eluted out in the first time segment (from 0 to 19 min) and switched to waste (by-passing the MS). The two minor breviflavones were completely separated from the major components and the internal standard.

There are many different *Epimedium* species reported [3,4]. *E. brevicornu*, *E. koreanum*, *E. pubescens*, *E. sagittatum*, and *E. wushanese* are the five major species [7]. Table 2 shows the content of the breviflavone A and B in the five major *Epimedium* species from different sources (Singapore, China, or Germany). As shown in Table 2, the different *Epimedium* species contain different content of breviflavone A and B. The contents of the two breviflavones in the same species from different sources were also different. The contents of the two breviflavones ranged from 0.0181 to 0.1791% for breviflavone A and 0.0026 to 0.0252% for breviflavone B in the dried ethanolic extracts of those *Epimedium* samples. Although measurement of breviflavone content would not clearly separate all *Epimedium* species, this LC–MS/MS method provides an excellent approach for rapid screening for bioactive breviflavone B from *Epimedium* species and herbal products. This type of chemical analysis can be used together with bioactivity profiling and genetic analysis for quality control of all *Epimedium* species as reported [3].

4. Conclusions

A robust and sensitive LC–MS/MS method for measurement of two new type of minor flavonoids, breviflavone A and B, in *Epimedium* herbal extracts has been developed. This method has been successfully used to determine the two breviflavones in the herbal extracts of five major *Epimedium* species (*E. brevicornu*, *E. koreanum*, *E. pubescens*, *E. sagittatum*, and *E. wushanese*) from various sources. The contents of the two breviflavones in different *Epimedium* species or in the same species from different sources are different for the five major *Epimedium* species.

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