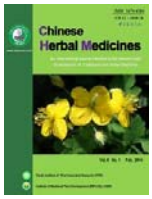

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Original article

# Rapid Detection of Acetylbritannilactone from *Inula britannica* in Plasma of Rats by Online Sweeping-Micellar Electrokinetic Chromatography

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ABSTRACT

**Objective** To establish a rapid method for detecting acetylbritannilactone (ABL) by online sweeping-micellar electrokinetic chromatography (MEKC) and to elevate the sensitivity of the detection. **Methods** The combination of online sweeping technique with MEKC was used to determine the content of ABL in the extract of *Inula britannica* in plasma of rats. **Results** ABL was completely separated within 15 min in running buffer and sample buffer. The optimal conditions were as follows: on uncoated fused quartz silica capillary, with separation voltage of 23 kV, capillary temperature of 25 °C, and detection wavelength of 195 nm. The regression equations revealed good linear relationships between the peak area and concentration of ABL ($r$ = 0.998), with the detection limits of 0.005–0.15 mg/mL. The relative standard deviations of migration time and peak areas for intra- and inter-batch were < 2.45% and < 2.26%, respectively. The recovery rate of this method was 96.3%–97.2%. **Conclusion** This method provides some advantages in separation speed, testing sensitivity, and operating convenience, with low sample and reagent consumption. The online sweeping-MEKC is an effective method for pharmacokinetic study and analysis on tracing biological samples.

*Key words*

1-*O*-acetylbritannilactone; *Inula britannica*; online sweeping-micellar electrokinetic chromatography; sesquiterpene lactone



## 1. Introduction

Traditional Chinese medicinal herbs, due to long time clinic experience and reliable therapeutic efficacy, are attracting global attention and serve as the excellent pools of bioactive compounds for the discovery of new drugs. In the past decades, a vast majority of active compounds have been discovered from these medicinal plants, such as camptothecin, taxol, vinblastin, vincristine, podophyllotoxin, colchicine with antineoplastic activities, and *Tripterygium wilfordii* multi-glycoside, simomenine, total glucosides of *Astragalus* showing anti-inflammatory activities, etc.

*Inula* L. from Compositae with more than 120 species is found mainly in Europe, Africa, and Asia. *Inula britannica* L. is a wild plant found in Eastern Asia, including China, Korea, and Japan. In traditional Chinese medicine, both *I. britannica*

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and *I. japonica* Thunb. are called *Xuanfuhua* in Chinese. The flowers have been used for the treatment of digestive disorders, bronchitis, and inflammation. The extracts have been reported to have anti-inflammatory, antibacterial, antihepatitic, and antitumor activities (Liu et al, 2004). Various sesquiterpene lactones have been isolated from the species of *Inula* L. in China, such as *I. britannica*, *I. japonica, I. salsoloides* (Turcz.) Ostenf., *I. hupehensis* (Ling) Ling, *I. helianthus-aquatica* C. Y. Wu ex Ling, and so on. Several sesquiterpene lactones have been shown to be inflammatory (Justin et al, 2013; Liu et al, 2009).

Recently, an extract from *I. britannica* was isolated and used to treat bronchitis and inflammation, and the extract contains acetylbritannilactone (1-*O*-acetyl-britannilactone, ABL) which belongs to sesquiterpene lactones (Liu et al, 2011). We found that ABL in the extract had a potent anti-inflammatory activity, and could inhibit NF-κB activitation and down-regulate the expression of iNOS and COX-2 genes in RAW 264.7 macrophages stimulated by LPS/IFN-γ (Liu et al, 2007; 2011). The powerful separation and sensitive detection of ABL are necessary for the quality control of the extract from *I. britannic*a and pharmacokinetic analysis of ABL. It has been known that ABL could be separated and analyzed through RP-HPLC method, but its sensitivity was a bit low (Wang et al, 2005). In the present study, a method for the determination of ABL in the extract of *I. britannica* in plasma of rats by micellar electrokinetic chromatography (MEKC) (Li et al, 2011) was established, in which online sweeping technique was applied to elevating the sensitivity of the detection. The results indicated that online sweeping-MEKC was a simple and rapid method for the determination of trace biological sample. The method needs only micro-amount of sample, and has higher automation and sensitivity.

## 2. Materials and methods

### *2.1 Apparatus and reagents*

P/ACETM MDQ Capillary Electrophoresis System, equipped with PDA Detector and MDQ Data Analyzer (Beckman Coulter, USA), was used for the detection. Fused silica capillary tube (total length 57 cm, effective length 50 cm, 75 μm) was purchased from Beckman Coulter, Inc. The analytical reagents including boric acid, borax, NaOH, methyl alcohol (of chromatographic grade), and sodium dodecyl sulfate (SDS) were all purchased from Sigma (USA). All chemicals were of analytical grade unless otherwise specified. The ABL (Figure 1) in the extract of *I. britannica* was separated and purified by Institute of Pharmaceutical Science of Hebei Medical University.

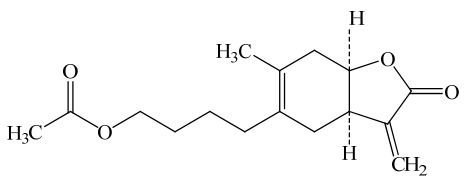


**Figure 1 Chemical structure of ABL**

### *2.2 Solution and sample preparation*

Running buffer was 50 mmol/L boric acid-borate buffer (pH 9.5) containing 50 mmol/L SDS. Sample buffer was 10 mmol/L boric acid-borate buffer (pH 9.5). The reference solutions of ABL were prepared in 6 mg/mL by adding ABL to sample buffer and were kept in 4 $^{o}$C refrigerator. The stock solutions were diluted to the desired concentration to be used. All the buffers, reference solutions, and sample solutions were filtered through 0.45 μm filter membrane before use.

Six SD rats were divided into control and drug groups with three rats in each group. The drug was ig given with 3 mL of the extract (containing 0.15 mg ABL) every day for 5 d. The blood was taken from the angular vein, centrifuged at 3000 r/min for 10 min to separate plasma after 5 d. The plasma protein was precipitated by adding equal volume acetonitrile to the blood. The precipitated substance was discarded. The supernatant was stored in −20 $^{o}$C refrigerator for use.

### *2.3 Procedure for sweeping-MEKC*

The detection wavelength was set at 195 nm and separating voltage was set at 23 kV. The samples were loaded on anode. The samples were injected by applying a pressure of 3.4 kPa for 5 s. The capillary temperature was maintained at 25 $^{o}$C. First, a pressure of 138 kPa was applied, then in turn, flushing with 0.1 mol/L HCl for 5 min, distilled water for 2 min, 0.1 mol/L NaOH for 5 min, and lastly flushing with running buffer for 5 min and balancing with running buffer for 10 min. Between any two operations, flushing was made with 0.1 mol/L NaOH and running buffer for 2 min, respectively.

### *2.4 Wavelength detection and peak purity*

A Diode Array Detector was used for scanning at 3-D full wavelength to decide the ideal detection wavelength that ABL peak was analyzed by spectrum to verify whether the detected peak had been caused by a single component.

### *2.5 Reproducibility test*

The rat plasma samples containing 0.015 mg/mL ABL were prepared and divided into 15 aliquots, of which 5 aliquots were grouped into one batch, and stored at −20 $^{o}$C. A batch was detected every 4 d. The reproducibility experiment was performed for three times for a batch. Each aliquot was repeatedly detected twice. The migration time of ABL was detected by the online sweeping-MEKC method. The average value and the relative standard deviation (RSD) were calculated within a batch and between batches.

### *2.6 Linear relationship and sensitivity test*

The rat plasma samples containing 1.5 mg/mL ABL were prepared and diluted to different concentration via multi-

proportion dilution. These samples were sequentially detected by the sweeping-MEKC method to verify the sensitivity of the established method and the linear relationship between peak area and ABL concentration.

### *2.7 Recovery test*

The rat plasma samples containing 0.15 mg/mL ABL were prepared and divided into six aliquots, 20 μL each. Three aliquots formed a group. ABL reference solutions (5 mg/mL, 1 and 3 μL) were added into the two groups, respectively. After mixing, acetonitrile of the same volume was added and mixed with a vortex for 30 s, and then centrifuged at 10 000 r/min for 10 min. The supernatant was tested via sweeping-MEKC. The recovery rate was calculated from ABL contents.

### *2.8 Determination of ABL in extract from I. britannica in plasma of rats*

The above established sweeping-MEKC method was applied to detecting the content of ABL in the extract from *I. britannica* in plasma of rats.

### *2.9 Statistical analysis*

The MDQ analysis software supplied with the P/ACETM MDQ Capillary Electrophoresis System was used to analyze the electrophoretogram. SPSS 10.0 statistical software was used to analyze other data.

## 3. Results

### *3.1 Determination of ABL*

By sweeping-MEKC method, the ABL content in the extract from *I. britannica* was detected, and the ABL peak was determined by comparison of capillary electrophoretogram of the extract from *I. britannica* with that of the ABL reference solution. Figure 2 is a typical electrophoretogram of the extract from *I. britannica*. After the extract from *I. britannica* was fed to rats for 0.5, 1.5, 3, 6, and 8 h, capillary electrophoretogram of the extract in plasma of rats was analyzed and ABL peak was determined by comparison with ABL solution reference. Figure 3 shows the electrophoretograms of ABL in the control and drug groups. Figure 4 is the electrophoretogram of the extract in plasma of rats after ABL was added. Figure 5 is the change curve of the ABL concentration in plasma of rats at different time administered with the extract of *I. britannica*. These electrophoretograms showed that the ABL peak could be detected in the drug group but not in the control group. This peak was greatly increased after ABL was added. Figure 5 shows that the ABL reached the maximum at 1.5 h after administration with the extract from *I. britannica*.

The optional conditions were as follows: uncoated fused silica capillary (57 cm × 75 μm), voltage of 23 kV, pressure

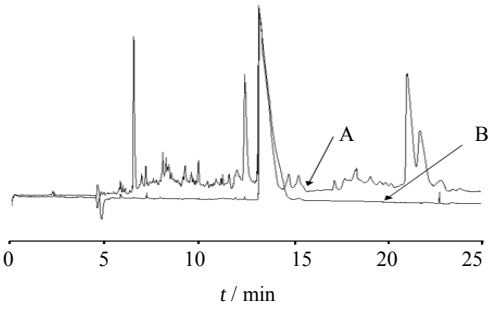


**Figure 2 Comparison on capillary electrophoretograms of extract from *I. britannica* (A) and reference ABL (B)**

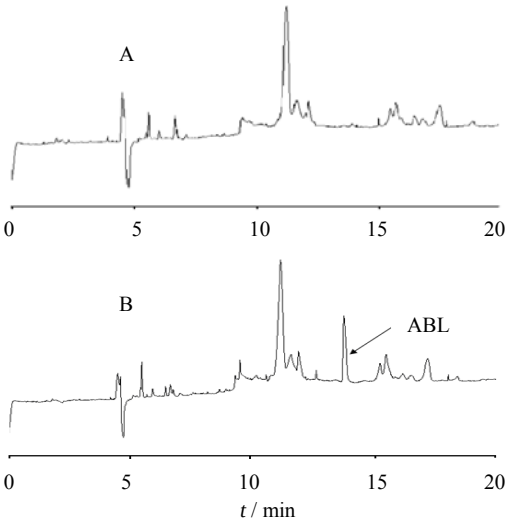


**Figure 3 Electrophoretograms of ABL in control (A) and drug (B) groups**

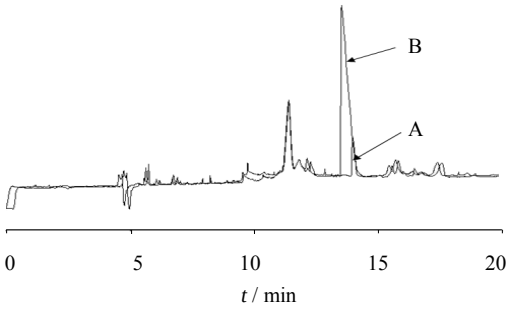


**Figure 4 Capillary electrophoretograms of extract from *I. britannica* in plasma of rats without (A) and with (B) ABL**

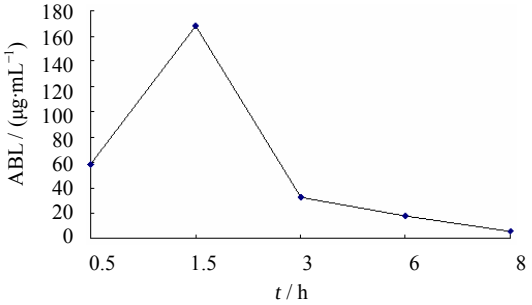


**Figure 5 ABL concentration in plasma of rats for different time administered with extract of *I. britannica***

injection of 3.4 kPa × 5 s, running buffer of 50 acid-borate buffer (pH 9.5) with 50 mmol/L SDS, sample buffer of 10 mmol/L boric acid-borate buffer (pH 9.5), detection wavelength of 195 nm, and capillary temperature of 25 °C. The ABL peak was detected at 13.36 min. The sweeping-MECC conditions are the same as those in Figure 2.

### *3.2 Methodological assessment*

The results of reproducibility experiment showed that the inter- and intra-batch variation coefficients were all less than 3% in the determination for each of the three sample batches (Table 1). The results of regression analysis on calibration curves and detection limits were as follows: The determination limits were evaluated on the basis of a signal-to-noise ratio (S/N) of 3. The calibration curves exhibited satisfactory linear behavior, and the linear regression equation of ABL concentration and peak area was $y = 925\ 054\ x + 18\ 760$, ($r = 0.998$) with the detection limits ranging from 0.005 to 0.15 mg/mL. The result of recovery test showed that the recovery rate of this method was more than 96%. The minimum testing limit, reproducibility, and recovery rate could meet the needs for testing trace amount of bioactive components.

**Table 1 Results of reproducibility test of ABL ($\bar{x} \pm s$, $n = 10$)**

| Batches | Groups | Migration time /min | RSD / % | | |
|---|---|---|---|---|---|
| | | | Intra-group | Intra-batch | Inter-batch |
| I | 1 | 13.33 ± 0.32 | 2.40 | 2.45 | |
| | 2 | 13.15 ± 0.23 | 1.75 | | |
| | 3 | 13.33 ± 0.43 | 3.23 | | |
| II | 1 | 13.15 ± 0.34 | 2.58 | 1.96 | |
| | 2 | 13.22 ± 0.28 | 2.12 | | 2.26 |
| | 3 | 13.40 ± 0.23 | 1.72 | | |
| III | 1 | 13.51 ± 0.25 | 1.85 | 2.32 | |
| | 2 | 13.40 ± 0.36 | 2.69 | | |
| | 3 | 13.56 ± 0.35 | 2.58 | | |

## 4. Discussion

### *4.1 Online sweeping-MEKC detecting technique*

Though there are many separation modes in capillary electrophoresis, MEKC mode is the only one that could simultaneously separate the neutral and charged substances. The principle of MEKC is to add surfactant to running buffer (Li et al, 2008). When the surfactant reaches its critical concentration, a flowing pseudo-stationary phase is formed in the running buffer. The neutral substance is distributed as difference of the distributing coefficient between the flowing phase and pseudo-stationary phase. Different charged substances are driven by the electromotive force and separated due to the type and quality of the charges they bear. In this way, the neutral and charged substances are separated. There are both charged and neutral substances in the extract of *I. britannica* in which ABL is a neutral liposoluble matter, thus MEKC method could be used. Like other capillary electrophoresis, the concentration responsiveness of MEKC is not high due to the limits of ultraviolet detector, detecting path length, and sample volume. Therefore, this technology is limited, to some extent, in detecting trace amount of substances. In order to improve the detecting sensitivity of MEKC method, this research attempts to combine the MEKC method with online sweeping technique for detecting the trace amount of ABL in plasma of rats (Natalia et al, 2010; Zhang et al, 2011).

Online sweeping technique (Cheng et al, 2011; Zhang et al, 2011; Su et al, 2008; Moreno-González et al, 2011) is a type of capillary electrophoresis concentrating mode that could be applied in MEKC. When a sample is enriched with sweeping, the sample band generally does not contain micelle, while the running buffer containing surfactant and acting as background micelle passes the sample band, the sample will move with the micelle due to mutual interaction between the sample and the micelle. As a result, the sample band is compressed and enriched. The ABL peak in plasma of rats could not be found when the sweeping enrichment technology is not used. The sample shows ABL chromatographic peak through sweeping enrichment. These results indicates that the combination of MEKC with online sweeping sample stacking technique effectively could improve the detecting sensitivity.

### *4.2 Sample preparation*

The blood of rats was taken from the angular vein, centrifuged at 3000 r/min for 10 min to separate plasma. The plasma protein was precipitated with trichloracetic acid, tungstic acid, methanol, and acetonitrile, respectively. It was demonstrated that acetonitrile had the best precipitating effect for the plasma protein. Therefore, acetonitrile was used to precipitate the protein. Acetonitrile of the same volume was added to 100 μL plasma and mixed with a vortex for 30 s, and then centrifuged at 10 000 r/min for 10 min. The supernatant was stored at −20 °C for use.

### *4.3 Optimal conditions for online sweeping-MEKC*

The experiment compared the different buffer systems including phosphate, tris-HAC, tris-boric acid, tris-phosphoric acid, and boric acid-borate. The results showed that the borate

buffer had the best separation effect in the buffers of the same ion strength. Thus, the borate buffer was selected in this experiment. Moreover, the effect of ion strength and sample loading buffer on the sample enrichment and separation was also explored. When the concentration of borate buffer was within 20–100 mmol/L, the resolution was increased with ion strength increasing but there was little influence on the sample enrichment. The boric acid-borate solution (50 mmol/L) could separate the sample on the base line.

Within the range of 20–80 mmol/L SDS, the sample enrichment was related with the increase in negative SDS concentration when 50 mmol/L boric acid-borate solution was used as running buffer. However, the negative SDS increased the amperage. The current rose considerably when the SDS concentration exceeded 50 mmol/L, and as a result, the base line moved up and the noise occurred. Therefore, this experiment selected 50 mmol/L boric acid-borate buffer containing 50 mmol/L SDS as the running buffer. Borate buffer of low concentration (10 mmol/L) was used as the sample buffer for online sweeping technique. In this way, the ABL peak area of the sample was increased by 125-fold.

With the separation voltage of 23 kV, 3.4 kPa × 5 s sample injection, and 0.015 mg/mL sample concentration, the influence of pH value of the running buffer on the sample enrichment was examined. The results showed that the sample peak reached the maximum at pH value of 9.5. Thus, pH 9.5 was selected for the running buffer.

The applied voltage had no significant effect on the sample enrichment, but the migration time was shortened, the current rose and the heat production increased when the voltage increased. In the present study, 23 kV was selected as the operating voltage.

Through the optimization of every experimental condition, ideal electrophoretic conditions were determined (see Procedure for sweeping-MEKC).

Although MEKC method could not be used to detect ABL in the plasma of rats, the combination of MEKC with online sweeping technique could greatly improve the detecting sensitivity, and make ABL peak evident. Under the optimal conditions, the detection limit of ABL was 0.005 mg/mL (S/N = 3), which was about 879 times lower than those in conventional sample injections. Through the wave spectrum analysis, it could be determined whether the detected peak was made up of single component, thus making the results more reliable. The present results indicated that sweeping-MEKC is a good method for pharmacokinetic study and analysis on biological samples.

In conclusion, this experiment established an online sweeping-MEKC method to detect ABL in the extract from *I. britannica* in plasma of rats. This method provides some advantages in separation speed, detecting sensitivity, operating convenience, and with low sample and reagent consumption. The present results indicate that sweeping-MEKC is an effective method for the quality control of the extract from *I. britannica* and its pharmacokinetic study. Online sweeping-MEKC is a good method for the pharmacokinetic study and analysis on trace biological samples. The method could make up for the shortcomings of detecting trace biological samples by capillary electrophoresis.

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