Aristolochic acids (1 and 2), a component in medicinal plants, have been reported as the cause of end-stage renal failure. For the identification and quantity determination of aristolochic acid 1 and 2 (AA-1 and AA-2) in *Radix Aucklandiae*, a liquid chromatography with electrochemical detection system was constructed in this paper. Both of the chromatographic peak heights for AA-1 and AA-2 at the detection potential of -0.8 V were found to be related linearly with the concentrations ranging from 10 ng/mL to 50 μg/mL. The limit of detection (S/N = 3) of AA-1 and AA-2 are 3.7 and 3.4 ng/mL, respectively.

**Keywords**: Electrochemical method; Aristolochic acid; *Radix aucklandiae*; Liquid chromatography;

**1. INTRODUCTION**

In the Chinese medicines, more than 500 examples of drugs extracted from plants are recorded. Among them, nearly 400 Traditional Chinese Medicines (TCM) are widely used throughout the world [1, 2]. Chinese herbs nephropathy (CHN) is a kind of disease resulted from fibrosis of kidney. The slimming regimen, which has been taken by some patients for weight-reducing, usually consists of Chinese herbs [3-6]. Due to the nephrotoxicity of Chinese herbs stem from aristolochic acid (AA), the renal replacement therapy (RRT) was demanded to support the patients [5-7]. Therefore, a ban to restrict the importation and sale of medicines containing aristolochic acid have been announced by US, UK, and many other countries over past 10 years [8, 9]. Aristolochic acid, a family of structurally related nitrophenanthrene carboxylic acids, was found in *Aristolochia* and *Asarum*. It has been found that more than 800 herbaceous or shrubby species involve the *Aristolochia* genus.
AA-1 (8-Methoxy-6-nitrophenanthro[3,4-d][1,3]dioxole-5-carboxylic acid) and AA-2 (6-nitronaphtho[2,1-g][1,3]benzodioxole-5-carboxylic acid) (structures shown in Figure 1) are the major components in Chinese herbs. In traditional Chinese medicine, significant confusion can possibly be caused by the same Chinese name shared by two different herbs. For example, the Aristolochia Fangji (Guang fang ji) containing aristolochic acid is often used by error, owing to Fangji is also frequently used as the name of the nontoxic herb Stephania tetrandra ST (Han fang ji). Therefore, developing an efficient method to detect the aristolochic acid is of great importance.

In the field of detecting aristolochic acid, high-performance liquid chromatography (HPLC) equipped with various detection modes [10-13] and capillary electrophoresis [14-16] have been widely used. The detection of AA-1 by HPLC with UV detection was reported by Chen et al. [17]. However, trace analysis of AAs could not be satisfied due to the poor sensitivity of UV detection. Fluorescence detector [18] as a substitute for UV detector was adopted to improve the detection sensitivity. Nevertheless, the detection is still difficult because most of AA molecules have no intrinsic fluorescence property. Except for the detection method mentioned above, mass spectrometry (MS) has been proven to be another effective detection method. The determination of AA-1 and AA-2 in Chinese medicinal plants using LC-MS method has been shown by Chan et al. [10]. Besides, the concentration of AA in the metabolites can be determined as well by LC-tandem MS (LC-MS-MS) [19]. As a whole, the LC-MS method is a sensitive and specific method for detecting AAs in drugs [19-23]. A capillary electrophoresis with electrochemical detection (CECED) in the oxidation mode at +1.2V vs. Ag/AgCl was applied to determine AA in Aristolochia plants [24]. However, many unknown peaks close to AAs peaks on electrophoregram were observed. Thus, electrochemical detection in the oxidation mode would be unsuitable for determining AA with high selectivity. The nitrophenyl group of AA is ideally suited to electrochemical detection in the reduction mode [25-27].

As a result, the HPLC-ECD (high-performance liquid chromatography with electrochemical detection) system, by means of the electrochemical reduction of AA, is expected to provide a sensitive, selective and accurate method for measuring the concentration of AA-1 and AA-2 in Chinese herbal medicines.

A stringent purity test for AAs and quality control of Chinese herbal medicine would be performed by the HPLC-ECD method in comparison with the existing HPLC methods. In our current work, a modified HPLC-ECD system to determine AA-1 and AA-2 with high sensitivity and selectivity was developed. The HPLC-ECD method was also applied to the detection of AA-1 and AA-2 in Radix aucklandiae.

Figure 1. Structures of AA-1 and AA-2.
2. EXPERIMENTS

2.1 Materials

Aristolochic acid 1 (AA-1), aristolochic acid 2 (AA-2), 3,5-di-tert-butyl-1,2-benzoquinone and methanol were purchased from Sigma Aldrich (USA). Other reagents used were of analytical grade. All chemicals were used without any purification after the purchase. The samples of the medicinal plants (Caulis Akebiae, Radix Aristolochiae Fangchi, Radix et Rhizoma Asari and Radix aucklandiae) with their roots were gathered from three stores in Zhengzhou. For each medicinal plant, 500 g sample was grated into powder and kept at -20 °C before use. Milli-Q water was used throughout the experiments.

2.2 Preparation of tested solution

The tested solution was prepared using the following method. Firstly, 30 ml of methanol was used to extract 1 g sample three times. After the methanol was evaporated, the residue was redissolved in 2 ml of methanol. Then the solution was passed through a PHP-LH-20 column (Amersham Pharmacia, Sweden) which was firstly rinsed with water and methanol. The aristolochic acid adsorbed in the column was eluted with 150 ml methanol containing 10 % acetic acid at 25 °C. The flow rate of flush fluid was 30 mL/min. After the evaporation of the solvent, the remaining solid was redissolved with methanol and filtered by an organic syringe filter. Some other methanol was added to ensure the volume of tested solution being 1 ml.

2.3 HPLC-ECD

The HPLC-ECD is comprised of a vacuum degasser (LC-26A, BAS, Tokyo), a pump (301M, Flom, Tokyo), an injector with a 5 μL injection loop (7125, Rheodyne, CA, USA), a microbore ODS column (Capcell Pak C18 UG120), a column oven (FT-1, BAS), and an electrochemical detector (LC-4C, BAS). The electrochemical cell (Radial flow cell, BAS) consisted of a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. The mobile phase was methanol-water-phosphoric acid (65:35:0.5, v/v/v). Other relevant parameters are set as follows: applied potential -0.7 V vs. Ag/AgCl, column temperature 40 °C and flow rate 25 μL/min. An internal standard method with 3,5-di-tert-butyl-1,2-benzoquinone as the IS was used for determination of the amount of AA-1 and AA-2 in the sample solution. The digital chromatogram data was recorded at sampling intervals of 0.1 s/point after the transformation of the analog data in real time by an A/D converter (Nihon Filcon Co. Ltd., Tokyo). Hydrodynamic amperometry measurements were done using an electrochemical analyzer. Prior to the measurement, the glassy carbon electrode was polished sequentially with 0.3 and 0.05 μm alumina paste and then rinsed well with water.
2.4 Recovery test

In order to prepare the tested samples, 5 ng AA-1 and 20 ng AA-2 were dissolved in the samples of various plant extracts. Meanwhile, the blank experiment was also carried out. Thus, the influence of constant error can be deducted from the data of tested sample. The recovery can be calculated by the obtained data divided by spiked amount.

3. RESULTS AND DISCUSSION

Reverse-phase chromatography with an ODS column was employed for detecting AA-1 and AA-2. A mixture of methanol-water solution containing 0.5% phosphoric acid was used as the mobile phase. For the sake of optimizing the applied potential for detecting AA-1 and AA-2 sensitively and selectively, hydrodynamic voltammograms for AA-1 and AA-2 with the concentration 5.0 μg/mL were measured. As shown in Figure 2, AA-1 and AA-2 were reduced at potentials of less than -0.1 V, and the reduction waves at -0.8 V were also shown in the hydrodynamic voltammograms. At applied potentials more negative than -1.2 V, both of the chromatographic peak heights and baseline noises increased. Therefore, to ensure a highly sensitive determination with good selectivity, the applied potential was set at -0.8 V in our work.

![Figure 2. Hydrodynamic voltammograms of AA-1 and AA-2.](image)

A typical chromatogram for AA-1 and AA-2 at the same concentration 10 μg/mL with 3,5-di-tert-butyl-1,2-benzoquinone (IS) is shown in Figure 3. The retention times of AA-2, AA-1, and 3,5-di-tert-butyl-1,2-benzoquinone were 11.2, 14.3, and 22.2 min, respectively. The resolution (Rs) between AA-2/AA-1 and AA1/DBBBQ were 3.6 and 5.7, respectively. The chromatographic peak heights of AA-1 and AA-2 were both found to be linearly related to the concentrations ranging from 10 ng/mL to
50 μg/mL. The correlation coefficients for AA-1 and AA-2 are 0.998 and 0.997, respectively. The standard solution with a concentration of 5.0 μg/mL was measured five times with the relative standard deviation (RSD) less than 3.4%. The detection limits (signal to noise ratio of 3) of AA-1 and AA-2 by a single injection were 3.7 and 3.4 ng/mL, respectively.

**Figure 3.** Chromatograms of standard solution of AA-1 and AA-2 by HPLC-ECD (5 μL of AA-1 and AA-2 was injected into HPLC-ECD at each concentration of 10 μg/mL).

The sensitivity of the HPLC-ECD method for determining AA-1 was compared with that of other available methods, and the results were shown in Table 1. It is firstly noticed that detection limit of presented system (3.7 ng/mL) is better than those of chromatography based methods. We attributed it to the high sensitivity of the coupled electrochemical component which are better than those listed in Table 1. Moreover, the determination of AA-2 using our proposed method also showed a superior analytical performance compared with that of β-cyclodextrin-modified capillary zone electrophoresis [28], 32P-postlabeling/polyacrylamide gel electrophoresis [29], capillary electrophoresis with laser-induced fluorescence detection [30] and high-performance liquid chromatography [31]. Therefore, the present HPLC-ECD is the best technique with highest sensitivity among all methods.

**Table 1.** Comparison of AA-1 detection using different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS</td>
<td>4.17 ng/mL</td>
<td>[32]</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>200 ng/mL</td>
<td>[33]</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>10 ng/mL</td>
<td>[34]</td>
</tr>
<tr>
<td>LC-MS</td>
<td>20 ng/mL</td>
<td>[35]</td>
</tr>
<tr>
<td>HPLC-ECD</td>
<td>3.7 ng/mL</td>
<td>This work</td>
</tr>
</tbody>
</table>

The nontoxic herb *Akebia quinata Decaisne* (Mu tong as the Chinese name) is normally used to produce *Caulis Akebiae*. Unfortunately, AA containing *Aristolochia manshuriensis Kom* (Guan mu tong) is often mistakenly used to produce *Caulis Akebiae*. Similarly, the nontoxic herb *Saussurea*
lappa Clarke (Mu xiang) are normally used to produce Radix Aucklandiae, but AA containing Aristolochia debilis Sieb. et Zucc (Qing mu xiang) and Aristolochia yunnanensis Franch (Nan mu xiang) are often mistakenly used. Firstly, our HPLC-ECD method was used to test the quantity of AA-1 and AA-2 contained as impurities in Caulis Akebiae and Radix Aucklandiae. The chromatograms were obtained by injecting 5 μL solution of the crude herbal drug Caulis Akebiae and Radix Aucklandiae. AA-1 and AA-2 in the Caulis Akebiae and Radix Aucklandiae were not detected by the HPLC-ECD method. Thus, considering the detection limits by our HPLC-ECD method, the concentrations of AA-1 and AA-2 were less than 67 and 71 ng/g in the Caulis Akebiae and Radix Aucklandiae studied, respectively.

The present HPLC-ECD system was also applied for the measurement of AA-1 and AA-2 in Radix Aristolochiae Fangchi and Radix et Rhizoma Asari. The chromatogram in Figure 4 was obtained by injecting 5 μL solution of the Aristolochiae Fangchi and Radix et Rhizoma Asari. Both AA-1 and AA-2 can be detected in the Radix Aristolochiae Fangchi and Radix et Rhizoma Asari. The concentrations of AA-1 and AA-2 in the Radix et Rhizoma Asari and Radix Aristolochiae Fangchi along with the recovery data were listed in Table 3. The concentrations of AA-1 and AA-2 were determined with RSD values of less than 4.21% (n = 5). Recovery tests of AA-1 and AA-2 were measured using standard AA-1 and AA-2 spiked in the Radix Aristolochiae Fangchi and Radix et Rhizoma Asari. The results were 98.1-101.3%, and the repeatability was less than 2.3% RSD (n = 5), demonstrating that the present HPLC-ECD method was adequate and precise for measuring AA-1 and AA-2 without being affected by other components. Radix et Rhizoma Asari is normally produced using a root and rhizome of Asiasarum sieboldii F. Maekawa which does not contain AA. Nevertheless, both AA-1 and AA-2 were detected in the crude herbal drug of Radix et Rhizoma Asari studied. It can be expected that an aerial part of Asiasarum sieboldii F. Maekawa is mixed to a root and rhizome part.

![Figure 4. Chromatograms of Radix et Rhizoma Asari obtained by HPLC-ECD](image.png)
Table 2. Detection of AA-1 and AA-2 in *Radix et Rhizoma Asari* and *Radix Aristolochiae Fangchi* using proposed HPLC-ECD.

<table>
<thead>
<tr>
<th>Name</th>
<th>Analysis</th>
<th>Detection (μg/g)</th>
<th>Addition (μg/g)</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Radix et Rhizoma Asari</em></td>
<td>AA-1</td>
<td>15.6</td>
<td>10</td>
<td>0.59</td>
<td>100.8</td>
</tr>
<tr>
<td></td>
<td>AA-2</td>
<td>19.9</td>
<td>20</td>
<td>4.21</td>
<td>98.1</td>
</tr>
<tr>
<td><em>Radix Aristolochiae Fangchi</em></td>
<td>AA-1</td>
<td>255.4</td>
<td>200</td>
<td>2.74</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>AA-2</td>
<td>686.2</td>
<td>1000</td>
<td>1.69</td>
<td>101.3</td>
</tr>
</tbody>
</table>

4. CONCLUSIONS

In this work, the HPLC-ECD method has been developed for the determination of AA-1 and AA-2. The method was proven to be sensitive and selective. The concentrations of AA-1 and AA-2 in *Radix et Rhizoma Asari* and *Radix Aristolochiae Fangchi* were determined precisely by the established HPLC-ECD system. Moreover, the signal responses of AA-1 and AA-2 in the real samples were found to remain stable for 3 months without polishing the glassy carbon working electrode surface. The performance of the HPLC-ECD system for separating AA-1 and AA-2 also remained stable for 3 months with the same column. Considering the detection limit by the present HPLC-ECD method, our method makes it possible to be a purity test, even when the concentration of AA-1 is less than XXX ng/mL in the sample. This value was calculated by dividing the detection limit of AA-1 by the HPLC-ECD method (XXX ng/mL) by the amount of crude herbal drug in test solution (XXX mg/mL). The reported HPLC-ECD herein, 10 times more sensitive in comparison to the official HPLC-UV, would be useful for detecting the presence of AA-1 and/or AA-2 in crude herbal drugs and health supplements.

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Reference


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