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Short communication

High-performance liquid chromatographic determination for aristolochic acid in medicinal plants and slimming products

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Abstract

A HPLC procedure with a silica gel RP-18 reversed-phase column for the determination of aristolochic acids I, II in medicinal plants and slimming products was developed. The mobile system 0.3% ammonium carbonate solution–acetonitrile (75:25, v/v) with pH 7.5 was the optimal buffer to clearly separate aristolochic acids I, II within 20 min. The recovery of aristolochic acids I, II in medicinal plants and slimming products was better than 90% by extracting with methanol and purifying through a PHP-LH-20 column. The major component was aristolochic acid I in *Aristolochia fangchi* and the level ranged from 437 to 668 ppm. Aristolochic acid II was the major component for *Aristolochia contorta* and its range was <1–115 ppm. Twelve out of 16 samples of slimming pills and powders contained aristolochic acids I and/or II. The major component in most slimming products was aristolochic acid II and the level ranged from <1 to 148 ppm. It may indicate that slimming products were not mainly made of *A. fangchi*.

Keywords: Aristolochic acid

1. Introduction

Chinese herbs nephrophathy (CHN) is a new type of subacute interstitial fibrosis of the kidney. In Belgium, there have been approximately 100 women patients who had followed a weight-reducing treatment that usually consisted of Chinese herbs and appetite suppressants (fenfluramine and diethylpropion), as well as cascara, belladonna extract, and acetazolamide [1–5]. About one-half of the patients needed the treatment of renal replacement therapy [3,6]. CHN is not limited to Belgium, but has been reported in other countries including France [7], Spain [1], UK [8] and Japan [9,10].

The nephrotoxicity of these Chinese herbs has been traced to aristolochic acid (AA), which is the major alkaloid extracted from *Aristolochia fangchi*, inadvertently included in the slimming pills [11]. AA is a mixture of structurally related nitrophenanthrene carboxylic acids, AA-I and AA-II being major components. Not only is AA nephrotoxic, it is also a potent carcinogen in laboratory animals [12,13]. In 1994, the first case of bladder cancer among the Belgian patients was reported [14]. By 1999, 40% of 19 kidney-biopsy specimens from a group of these patients showed multifocal, high-grade, flat, transi-

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tional-cell carcinoma in situ [15]. Hence, the relationship between the use of *A. fangchi* and the development of urothelia carcinoma is not a minor problem. According to these facts, Belgium, UK, Canada, Australia and Germany have banned the use of these herbs.

Usually, slimming regimens consist of two herbs *Stephania tetrandra* and *Magnolia officianlis*. Among them, *S. tetrandra* is often substituted by *A. fangchi* because the Chinese names sound similar. These slimming pills and powders including different species *Aristolochia* are commonly used in Taiwan. Meanwhile, these species of *Aristolochia* are also used to act as analgesic, diuretic and antiinflammatory agents in Taiwan. Although the incidence of CHN in Taiwan is not officially reported, this problem of CHN is noteworthy here.

The quantitative analysis of AA in the medicinal plants has recently developed by using high-performance liquid chromatography (HPLC) [16]. However, this method took about 40 min for a run. Hence, this study was carried out to establish a simple, rapid, sensitive and usable method for determining AA-I and AA-II. Then the levels of AA-I and AA-II in medicinal plants and slimming pills and powders collected from herbal stores and patients in Taiwan were analyzed.

2. Materials and methods

2.1. Agents

A mixture of AA-I (61%) and AA-II (19%) was purchased from Sigma (lot 36H13111) (St. Louis, MO, USA) and further purified to obtain pure AA-I and AA-II standards by HPLC. The purified AA-I and AA-II were desalted by adding acetic acid and then evaporated. AA-I and AA-II were identified by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS) in this laboratory. The structural features of AA-I and AA-II were the same as those reported previously [17]. The purity of AA-I and AA-II was more than 99%. Methanol (LC grade) and other reagents were from Merck (Darmstadt, Germany).

2.2. Materials

The root (five samples) of the medicinal plant *A. fangchi* and the seeds (six samples) of the medicinal plant *A. contorta* were collected from six medicinal plant stores in Keelung City, northern Taiwan. Each sample was about 300 g. Each sample was immediately ground into powder and stored at −20°C before use. Furthermore, five slimming pills and 11 slimming powders were collected from victims who have followed the slimming regimen for some time and have been diagnosed to suffer slight renal failure in the Veterans Division of the General Hospital, Taipei. These slimming products were ground, mixed well, and then stored at −20°C for use. The moisture of the samples ranged from 10 to 15%.

2.3. Preparation of tested solution

The sample (1 g) was extracted with 15 ml of methanol thrice. After evaporation of solvent the residue was dissolved in 1 ml of methanol and the solution was applied to a PHP-LH-20 column (1.5× 2 cm I.D.) (Amersham Pharmacia, Sweden). Elution was carried out at a flow-rate of 30 ml/min at 25°C. The column was first rinsed with 20 ml of water and 40 ml of methanol. Then the AA fraction was eluted with 130 ml of 10% acetic acid in methanol. The AA fraction was evaporated in vacuum to dryness and the residue was dissolved with a small volume of methanol, filtered through a poly(vinylidene fluoride) (PVDF) syringe filter (National Scientific Company, USA) and added methanol to 1 ml. The filtrate was submitted to HPLC as described below.

2.4. Recovery test for AA-I and AA-II added to medicinal plant

The tested samples were prepared by dissolving 5 and 20 ng of the mixture of AA-I and AA-II in samples of *A. fangchi*, *A. contorna* and slimming products. A tested sample was extracted with methanol, applied to the PHP-LH-20 column as described in the previous section and then determined by using HPLC as described below. In this experiment, the blank test (no addition) was also done. The data of tested samples were subtracted from those of blank

samples. Then the obtained data were divided by spiked amount to calculate the recovery.

2.5. Instrumentation

AA-I and AA-II were determined by using a Hitachi liquid chromatograph (Tokyo, Japan) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector set at 254 nm, and a Model D-2500 chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 25×0.3 cm I.D., E. Merck) was used for separation.

2.6. Chromatographic conditions

The mixed solvent of 0.3% ammonium carbonate solution–acetonitrile was used as mobile phase. The optimal ratio of 0.3% ammonium carbonate and acetonitrile was first determined to separate AA-I and AA-II. Then the optimal pH of the mobile phase was studied by using 0.1 *M* sodium hydroxide and 0.1 *M* hydrogen chloride. The flow-rate was 0.8 ml/min.

2.7. Standard curves

The stock standard solution was prepared with methanol by dissolving 12.5 mg of a mixture of AA-I and AA-II into 100-ml volumetric flasks, and adding methanol to a final volume of 100 ml. Then 1 ml of the stock standard solution was diluted with methanol to obtain the tested standard solution (true concentration being 1 ppm for AA-I+AA-II). Standard curves of AA-I (0.1–1.9 ng) and AA-II (0.1–2.5 ng) were separately prepared and the peak area (y) vs. amount of AA (x) was plotted. Data for standard curves were subjected to linear regression analysis.

3. Results and discussion

The effect of acetonitrile concentration on the retention time of eluting peaks of AA-I and AA-II in HPLC by using the buffer (pH 9.3) prepared from different ratios of 0.3% ammonium carbonate solution and acetonitrile is shown in Fig. 1. The optimal ratio of 0.3% ammonium carbonate solution–acetonitrile was 75:25 (v/v) for rapidly separating AA-I and AA-II with good resolution in HPLC. The respective retention times of AA-I and AA-II were 9.0 and 6.9 min, but it was 32.6 min for AA-I and 22.9 min for AA-II by using acidic buffer as elution phase [16]. AA-I and AA-II are acidic compounds (Fig. 2). The ionization of both compounds in alkali solution increased the hydrophilic ability so the retention time was decreased when both compounds were eluted by buffer on a reversed-phase column.

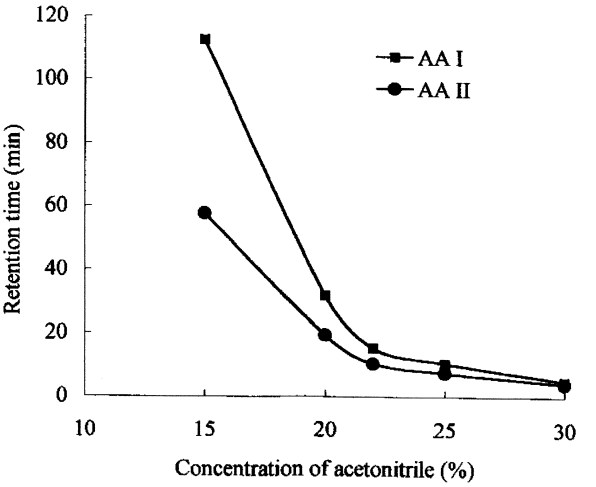
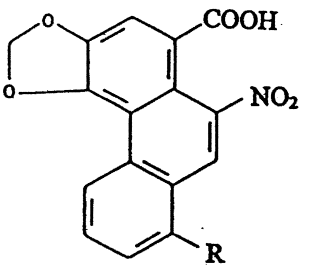


Fig. 1. Effect of acetonitrile concentration on the retention time of aristolochic acids I, II in HPLC by using the buffer (pH 9.3) prepared from different ratios of 0.3% ammonium carbonate solution and acetonitrile.



Aristolochic acid I : R= OMe
Aristolochic acid II : R= H

Fig. 2. Structures of aristolochic acids I, II.

Effect of pH value on retention times of elution peaks of AA-I and AA-II in HPLC by using 0.3% ammonium carbonate solution–acetonitrile (75:25, v/v) buffer is shown in Fig. 3. The optimal pH value in eluting buffer was 9.3 to shorten the retention time. However, high pH (9.3) in the eluent shortened the separation times of AA-I and AA-II, it might also destroy the stability of resin and shorten the column life. Lower pH (7.5) might be another suitable separating condition for lengthening the column life. Between pH 8.0–9.0, the $-COO^-$ and $-NO_2$ groups of ionized AA-I and AA-II might form new cyclic ring, or ionized AA-I and AA-II polymerized themselves. Hence the ionization of AA-I and AA-II was decreased and the retention time was lengthened. Once the buffer was pH 9.3, the new cyclic ring or polymerized compound was destroyed. AA-I and AA-II became more ionic. With regard to the analysis time, resolution and column stability, we suggested that the optimal eluting buffer was 0.3% ammonium carbonate solution–acetonitrile (75:25, v/v) with pH 7.5 for separating AA-I and AA-II in HPLC. Using this eluting system, a typical chromatographic profile of standard AA-I and AA-II is shown in Fig. 4. Both components were well separated in a 20-min total run time with good peak resolution, sharpness and symmetry. The recoveries of these two components from medicinal plants and slimming products were better than 90% (Table 1). It indicated that the extraction and purification procedure was suitable for extracting AA-I and AA-II. The correlation coefficients (r) and linear regression coefficients were as follows: AA-I, $y=0.1147x-0.0473$ ($r=0.9984$) and AA-II, $y=19.4510x+0.2661$ ($r=0.9987$). The correlation coefficient in each curve was better than 0.99. This indicated a definite linear

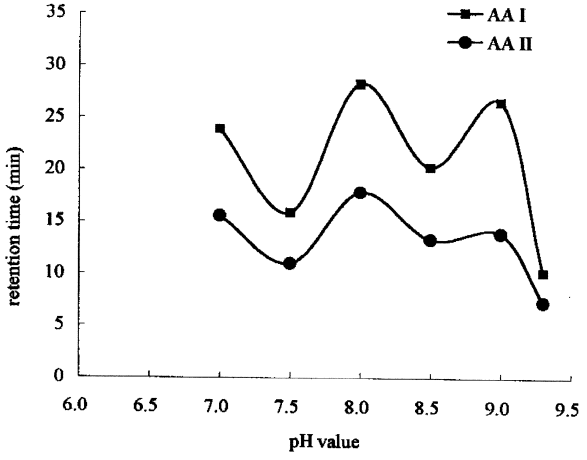


Fig. 3. Effect of pH value on the retention time of aristolochic acids I, II in HPLC.

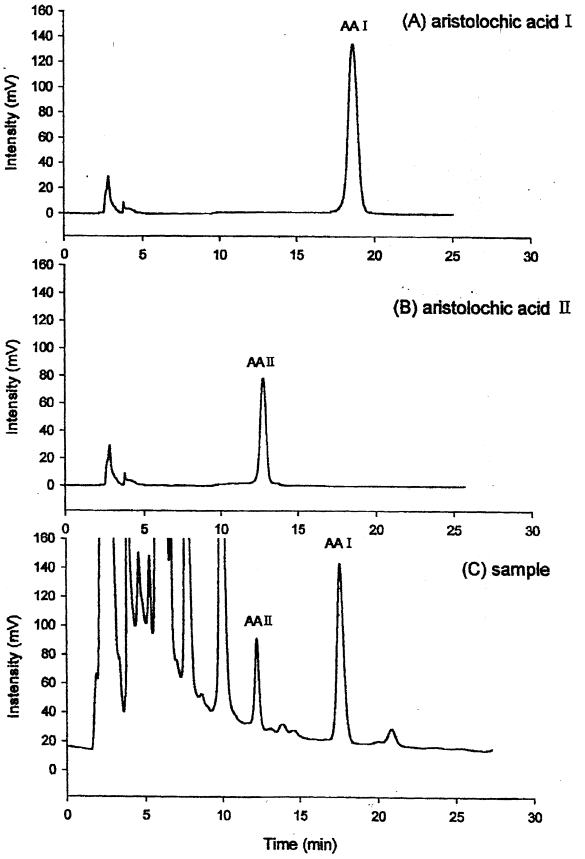


Fig. 4. HPLC profiles of Chinese medicinal slimming powder (sample 11) and authentic acids I, II.

Table 1
Recoveries of aristolochic acid (AA-I) and AA-II spiked into the Chinese medicinal plants and slimming products

Sample	Recovery (%) in different spiked dose		
	5 ng	20 ng	Average
Aristolochia fangchi	92.3±5.1*	89.4±3.0*	90.9
Aristolochi contorta	93.2±2.1	90.3±3.6	91.8
Slimming pill	91.8±3.4	90.2±3.8	91.0
Slimming powder	93.5±2.9	91.3±4.1	92.4

*Data represent mean±SD ($n=5$).

relationship between AA concentration and detector response. The detection limit for both compounds was found to be 0.1 ng (RSD values being less than 5%, $n=5$).

The typical HPLC profiles of AA-I and AA-II in medicinal slimming product are shown in Fig. 4. The identity of AA-I and AA-II peaks in the sample was not only confirmed by the retention time, but also re-confirmed by adding standards in the sample to show the same peak. The levels of AA-I and AA-II in two medicinal plants and slimming products are summarized in Table 2. The level of AA-I was higher than that of AA-II in *A. fangchi*. However, the level of AA-I was usually lower than that of AA-II in *A. contorta*, slimming pills, and slimming powders. The total amount of AA-I and AA-II in *A. fangchi* was usually more than fivefold of that in *A. contorta* and slimming products. Therefore, the slimming products sold in Taiwan may not be made of *A. fangchi*.

Table 2
The levels of aristolochic acid (AA-I) and AA-II in the samples of Chinese medicinal plants and slimming products

Sample	Level (ppm)		
	AA-I	AA-II	AA-I+AA-II
Aristolochia fangchi			
1	456±10*	144±5*	600
2	547±10	188±6	735
3	668±12	198±9	866
4	451±10	257±11	708
5	437±12	414±18	851
6	548±22	412±21	960
Aristolochia contorta			
1	83±6	115±9	198
2	<1	<1	
3	42±7	99±8	141
4	<1	<1	
5	60±8	101±8	161
Slimming pill			
1	24± 1	93±5	113
2	39± 3	124±5	163
3	28±1	<1	28
4	<1	<1	
5	<1	<1	
Slimming powder			
1	26±1	79±7	105
2	<1	94±7	94
3	<1	<1	
4	<1	76±3	76
5	23±1	78±5	101
6	<1	<1	
7	24±2	95±8	119
8	22± 1	<1	22
9	22±1	<1	22
10	30±2	148±9	178
11	598±5	94±4	694

*Data represent mean±SD ($n=2$).

By analogy, Hashimoto et al. [16] reported that the ranges of AA-I and AA-II levels were 1030–2220 ppm and 40–220 ppm, respectively in *A. fangchi* collected from Tokyo. The variation in the levels of AA-I and AA-II in the medicinal plants would depend on species, regionality and seasonality. Although the evidence of an association between the use of a Chinese herb, *A. fangchi*, and CHN and urothelial cancer in a group of Belgian patients has been reported [2,3,11,18], the level of AA-I and/or AA-II in the slimming regimens taken by those patients is unknown so far.

Furthermore, the use of medicinal plants of *Aristolotia* is widespread in the Orient. While the incidence of CHN in this area is rarely reported. The possible reason is the use of other herbs to minimise the potential side effects of *Aristolochia* [19]. As the major component in *A. fangchi* was AA-I, but AA-II in *A. contora*, the different side effects of AA-I and AA-II need further study. Besides AA-I and AA-II, the *Aristolochia* plants contain other AA-related compounds [20–22], these AA-related compounds are also require further study of their side effects. The effect of pH on retention times of AA-I and AA-II in HPLC exhibited unique curves, the reason is not known. There might be some relation with the interaction of $-COO^-$ and $-NO_2$ of AA under alkali condition to undergo cyclization.

4. Conclusion

A HPLC system using a mobile phase of 0.3% ammonium carbonate solution–acetonitrile (75:25, v/v) with pH 7.5 in combination with a C_{18} column was useful for determining aristolochic acids I and II. The major component was aristolochic acid I in the medicinal plant *A. fangchi*, but aristolochic acid II in *A. contora* and slimming products. The profile of aristolochic acids was quite different depending

on the plant species. The slimming products collected in Taiwan may not be mainly prepared from *A. fangchi*.

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