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Occurrence of Aristolochic Acids in Over-the-counter Chinese Prepared Medicines

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The purpose of the present study was to investigate the levels of aristolochic acids (AA-I and AA-II) in Chinese herbal medicines and in over-the-counter Chinese prepared medicines using high-performance liquid chromatography (HPLC). Reversed-phase HPLC (C_{18}) utilizing isocratic elution (65% methanol, 35% water, 0.5% acetic acid) and UV detection ($\lambda = 254$ nm) was used to survey 11 different kinds of Chinese herbal medicines that were known to be consumed by patients prior to be hospitalized for acute renal failure. It was found that 8 out of 11 samples contained aristolochic acids (ranging from 0.2 to 20 nmol/g. These results suggested that commercially available over-the-counter Chinese herbal medicines might contain various levels of aristolochic acids.

Key Words: Aristolochic acid, HPLC, Chinese herbal medicine.

INTRODUCTION

In July 1999, two cases of nephropathy, associated with the use of Chinese botanical preparations, were reported from the United Kingdom. Both of these patients had taken botanical preparations for the treatment of "eczema". These botanical preparations were determined to contain aristolochic acid, a known nephrotoxin, which can be found in *Aristolochia* spp., *Asarum* spp., *Bragantia* spp., *Stephania* spp., *Clematis* spp., *Akebia* spp., *Cocculus* spp., *Diploclisia* spp., *Menispernum* spp., *Sinomenium* spp., Mu Tong, Fang ji, Guang Fang ji, Fang Chi and Akebiae ('Mokutsu' in Japanese). Biopsy samples from both patients showed extensive loss of cortical tubules with interstitial fibrosis, features typical of the nephropathy referred to as "Chinese herbs nephropathy" (CHN). In 2000, Nortier *et al.*[2] reported that aristolochia toxins (aristolochic acids and also other possible derivatives) cause renal disease and urothelial cancer, which has a high prevalence among patients with end-stage CHN[2]. CHN has also been reported in a woman

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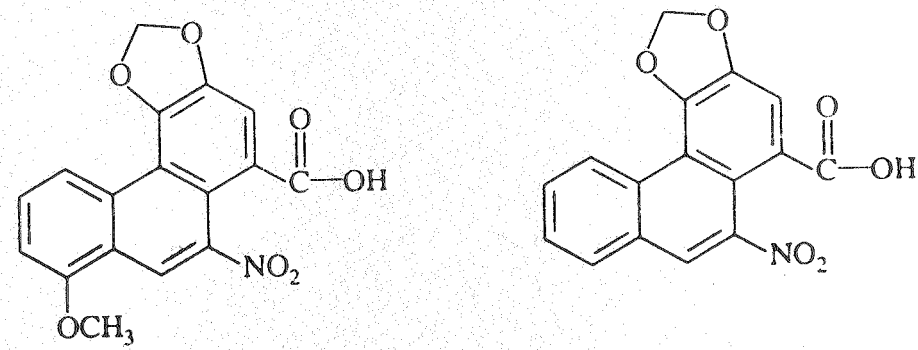
after the intake of weight-reducing pills containing *Stephania tetrandra*[3], which was identified to contain aristolochic acids (AA). AA are known for their nephrotoxic effects in rodents[4]. Such an observation was reported by Debelle *et al.*[5] group; specifically that AA induced chronic renal failure with interstitial fibrosis in rats, which is the typical histological finding initially observed in the renal superficial cortex. Exposure to AA was also confirmed by the detection of AA-DNA adducts in kidney tissue samples[1-6].

In a letter from the FDA to industry, it was stated[7] that in recent years, there have been several reported instances of severe nephropathy in consumers consuming products containing aristolochic acids. The incidents range from a few cases to, in one incident in Belgium, over 100 cases of nephropathy, linked to the use of a herbal product containing aristolochic acids.

Studies have reported the carcinogenic effect of aristolochic acids as well as many clinical cases of renal failure and acute hepatitis and this has given rise to research interest in the analysis of aristolochic acids in the Chinese medicinal herb and Chinese prepared medicine[7-12]. Aristolochic acid I (AA-I) and II (AA-II) (Fig. 1), which are found naturally in medicinal plants, such as *Radix aristolochiae*, have been reported for years in the Pharmacopoeia of People's Republic of China for use in relieving pain and inducing diuresis[13].



Aristolochic acid I Aristolochic acid II

Fig. 1. Chemical structures of aristolochic acid I (AA-I) and II (AA-II)

Several analytical approaches have been used to analyze aristolochic acids. Among them are solvent extraction followed by TLC[14], ultrasonic extraction followed by HPLC analysis[14,15] and pressurized liquid extraction (PLE) followed by capillary zone electrophoresis[16]. The purpose of the present study was to develop a more efficient procedure for sample preparation with shorter time and less organic solvent used. In addition, the contents of AA-I and AA-II were analyzed quantitatively in 11 different kinds of Chinese herbal medicines that were known to be commonly consumed by patients prior to hospitalization for acute renal failure. The proposed procedure involved extraction of aristolochic acid with MeOH. Chromatographic separation was carried out with a mobile phase of 65% methanol, 34.5% water, 0.5% acetic acid on a reversed-phase HPLC (C_{18}) column.

EXPERIMENTAL

Aristolochic acid I and II are potentially carcinogenic and mutagenic materials that should be handled with care. Crystalline aristolochic acid I, II and organic solvents for use in the extraction and HPLC analysis were handled in a chemical hood with surgical gloves.

Aristolochic acid I and II, glacial acetic acid, HPLC-grade water and methanol and all other chemicals and solvents were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO). Nylon filters (0.45 μm) were obtained from Rainin (Woburn, MA). Whatman No. 4 filter paper was bought from Fisher Scientific (Fair Lawn, NY).

Over-the-counter (OTC) Chinese prepared medicines including Mahuang and Cimicifuga Combination were purchased from a local store between January and September 2001. A total of 11 samples were analyzed in this study.

HPLC system

A reversed-phase liquid chromatograph equipped with a UV detector was used in this study. The HPLC system consisted of an isocratic pump (BAS model PM-80, Bioanalytical System, Lafayette, IN), an on-line degasser (CMA model 260, Solna, Sweden), a Rheodyne injector (model 7725) (Rainin, Emeryville, CA), an Econosphere C_{18} analytical column (15 cm × 4.6 mm, 5 μm particle size) (Alltech, Deerfield, IL) and a column oven set at 35°C to allow increased efficiency of column separation. For the detection of absorbance at 254 nm, an Alltech model 450 UV (Deerfield, IL) was employed. Data collection and analysis were performed with a Chem Station Chromatographic Management system (Hewlett-Packard, Taiwan Branch, Taipei, Taiwan). The LC mobile phase consisted of 65% methanol, 34.5% water, 0.5% acetic acid. Mobile phase was filtered through 0.2 m Nylon-66 filters and pumped at 1 mL/min. The mobile phase was freshly prepared each day.

Standard solution

A mixture of AA-I (43%) and AA-II (54%) purchased from Sigma (lot. 100K 1212) was used as standard. The stock solution was prepared to contain 0.32 mM AA-I and 0.44 mM AA-II in MeOH. Dilutions were prepared from this stock solution for the optimization and calibration studies.

Analytical sample preparation

A modified extraction method was used to recover AA-I and AA-II. 30 g of powdered sample was dissolved in 15 mL methanol and extracted by vortexing, followed by sonication for 10 min. After removal of the supernatant by filtration through fluted Whatman No. 4 filter paper, the dry residue was extracted twice more with MeOH (15 mL, 10 min and 10 mL, 10 min). The pooled supernatants were collected in the sample vial and evaporated to dryness under a stream of nitrogen. The collection vials were rinsed with an additional 0.4 mL of 50% MeOH and filtered (0.45 μm pore size) to give the analytical sample for HPLC. A sample (20 μL) was injected as well as standard solutions. The samples were stored in a capped vial at 4°C until HPLC analysis.

LC analysis

All procedures were based on Tanaka *et al.*[10] and Hashimoto *et al.*[14].

HPLC analysis

The samples were analyzed by a reversed-phase, isocratic HPLC system described above and used in an isocratic mobile phase consisting of 65% methanol, 34.5% water, 0.5% acetic acid. The injector, equipped with a 20 µL sample loop, was used to inject syringe-measured 20 µL aliquots on to the HPLC column. The programmable UV detector was set at an absorbance wavelength of 254 nm for the detection of aristolochic acids. The concentration in the extracts of aristolochic acid I and II was determined from a standard curve, using integrated peak area for quantification. If the sample concentration was above the calibration points on the standard curve, the sample extract was diluted quantitatively with HPLC mobile phase and reinjected.

RESULTS AND DISCUSSION

Various mobile phase compositions were examined for separating aristolochic acids. The most suitable mobile phase, enabling the system to give a fast separation of AA-I and AA-II with minimum interference, consists of 65% methanol, 35% water and 0.5% acetic acid. A typical HPLC chromatogram obtained from the separation of AA-I and AA-II of a standard solution is shown in Fig. 2. The method is fast as the analysis requires less than 9 min to complete. A shorter analysis time may be achieved by using a higher flow rate of 1.2 mL/min or a shorter C_{18} analytical column (10 cm length). Several OTC Chinese prepared medicines were bought locally and analyzed for aristolochic acids, AA-I

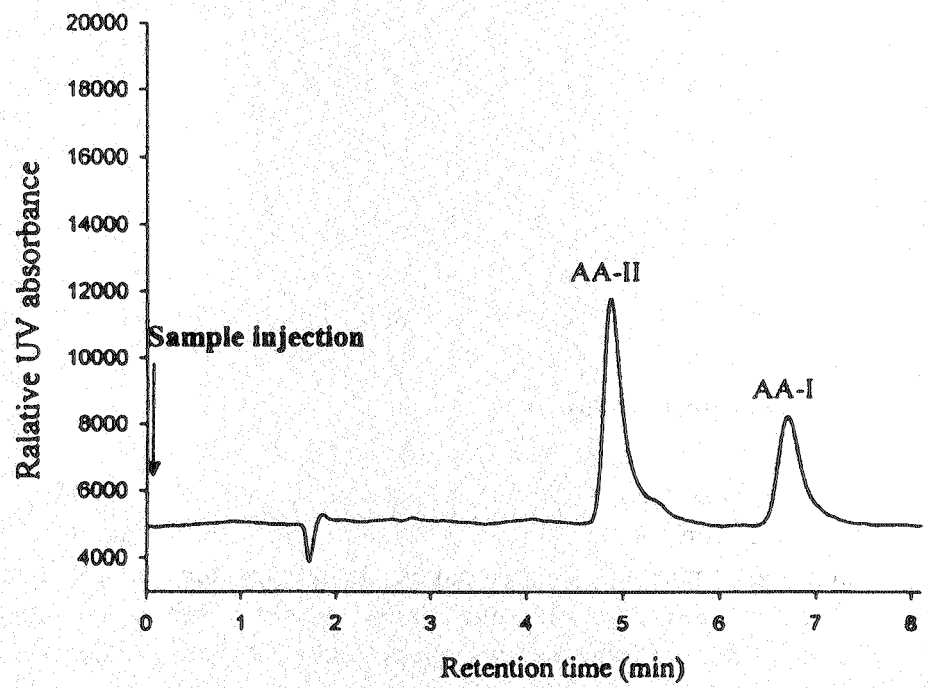


Fig. 2. A typical chromatogram of the aristolochic acids standards. The mobile phase consists of 65% methanol, 34.5% water, 0.5% acetic acid; flow rate, 1 mL/min; injection volume, 20 µL; detection, UV at $\lambda = 254$ nm

and AA-II. The results of the quantitative analyses are summarized in Table-1 and this indicates the aristolochic acid levels in these commercial OTC Chinese prepared medicines. 8 out of the 11 tested samples showed a measurable level. The quantitative analyses obtained from the present study demonstrated that the content of both AA-I and AA-II in OTC Chinese prepared medicines varied from non-detectable to 19.967 nmol/g for AA-I and from non-detectable to 3.952 nmol/g for AA-II based on duplicate determinations. In the Mahuang and Cimicifuga Combination medicine, there was 2.967 nmol/g and 3.952 nmol/g of AA-I and AA-II, respectively. Mahuang and Cimicifuga Combination is a common self-medication Chinese prepared medicine, which is usually taken to relieve the symptoms of common cold such as headache, fever, no perspiration, coughing, severe chills after catching a cold, nasal congestion, hoarse voice from the common cold, a stifling sensation in the chest and aversion to cold. The medicine is made up from 8.34% of *Pueraria radix* (Ge Gen), 8.34% of *Cimicifuga foetida rhizoma* (Sheng Ma), 8.34% of *Ligusticum rhizoma* (Chuan Xiong), 8.34% of *Angelica dahurica radix* (Bai Zhi), 8.33% of *Ephedra herba* (Ma Huang), 8.33% *Perilla folium* (Zi Su Ye), 8.33% of *Glycyrrhiza radix* (Gan Cao), 8.33% of *Citrus reticulata* (Chen Pi), 8.33% of *Cyperus rhizoma* (Xiang Fu), 8.33% of *Paeonia rubra radix* (Chi Shao), 8.33% of *Zingiber officinale radix* (Sheng Jiang) and 8.33% of *Allium fistulosum bulbus* (Cong Bai).

TABLE-1
CONTENTS OF AA-I AND AA-II IN OTC CHINESE PREPARED MEDICINES

S. No.	AA-I (nmol/g)	AA-II (nmol/g)
1.	4.847	1.144
2.	19.970	0.481
3.	0.666	0.846
4.	ND	ND
5.	7.621	2.589
6.	ND	ND
7.	0.204	ND
8.	ND	ND
9.	5.408	ND
10.	4.949	ND
Mahuang and Cimicifuga Combination	2.967	3.952

ND, Not detectable, the amount of AA-I and AA-II in the tested portion is less than the detection limit of 0.067 nmol/g for aristolochic acids.

Conclusion

A simple, fast HPLC method for the analysis of aristolochic acids in over-the-counter Chinese prepared medicines was developed using UV detection. Time and labour-consuming sample cleanup procedure or pretreatment was not

required. Several screening studies of aristolochic acids in Chinese prepared medicines have reported high levels of AA-I and AA-II in tested samples[10, 14]. In this study, 8 out of 11 samples were found to have detectable amount of AA-I or AA-II. These results suggested that certain over-the-counter Chinese herbal medicines may have a very high chance of containing some but variable levels of aristolochic acids. Due to their potential harmful effects, the aristolochic acids-containing Chinese herbal medicines should be used for self-medication with particular precaution. It is also essential that the medicinal plants used for Chinese traditional medicines should be strictly identified based on botanical as well as chemotaxonomic information.

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