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Study on Solid-Phase Extraction and Ultra Performance Liquid Chromatography for the Determination of Aristolochic acid A in Herbal Plants

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A solid-phase extraction and ultra performance liquid chromatography for the determination of aristolochic acid A in herbal plants was studied in this manuscript. The aristolochic acid A was extracted from samples by 33 % methanol (containing 2 % formic acid). The extraction was purified by strong anion-exchange reversed-phase extraction cartridge (Oasis MAX extraction cartridge). Some interferences could be removed from the samples by this procedure. Under optimum condition of ultra performance liquid chromatography-diode array detection (UPLC-PDA), a baseline separation was achieved for aristolochic acid A within 7 min. The linearity of aristolochic acid A was in the range of 32.27-1236 ng/mL. The detection limit (S/N = 3) was 1.65 ng/mL for aristolochic acid A. The relative standard derivation of overall intra-day variations were less than 2.0 % and the relative standard derivation of inter-day variations were less than 2.5 %. The average recovery factors were ranged from 91-102 %. This method is simple, rapid and sensitive to detect aristolochic acid A in herbal plants.

Key Words: Aristolochic acid A, Solid phase extraction, Ultra performance liquid chromatography.

INTRODUCTION

Aristolochic acids A (AAA) have been found to be nephrotoxic, carcinogenic and mutagenic[1-5]. The herbal plants and their preparations containing aristolochic acid A have been prohibited in most of the countries. However, some drugs with lower contents of aristolochic acid A still can be found in markets[6]. For drug safety, it is necessary to develop a sensitive and rapid analysis method which can confirm the absence of aristolochic acid A in any drug products.

A series of literatures, including UV-spectrophotometry[7], polarography[8], capillary electrophoresis (CZE)[9], thin layer chromatography (TLC)[10], high-performance liquid chromatography coupled to DAD detection (HPLC-DAD), UV detection (HPLC-UV)[11] or mass spectrophotometry (LC-MS)[4] have been reported for the determination of aristolochic acid A in aristolochiaceae.

Among them, the HPLC method has the advantage of detection of aristolochic acid A as simple operation. However, using ultra performance liquid chromatography (UPLC), the separation time was shortened, and reducing the run time by 7.5 min and a better resolution was achieved compared to the routine HPLC. Due to higher peak efficiency achieved with ultra performance liquid chromatography, despite the fact that in ultra performance liquid chromatography twice lower sample volumes were injected. Satisfactory and comparable recoveries (97-102 %) were obtained by ultra performance liquid chromatography for all samples. Ultra performance liquid chromatography gave significantly better precision and lower solvent consumption.

Ion interaction as a primary retention mechanism of ion-exchange solid-phase extraction, the ion interaction was occurs when the analysis of molecules with a positive or negative charge, but the stationary phase with the opposite charge. There are two types used ion-exchange stationary phase, a positively charged or cationic compounds can be retained with cation-exchange phase; according to change the pH, organic amines and carboxylic acid that does not take charge can ionized. Anion-exchange phase and cation exchange relative to the contrary, it retains the negatively charged or anionic compounds. The aristolochic acid A belong to carboxylic acid, so the solid phase extraction with strong anion-exchange reversed-phase can be used to retain aristolochic acid A with high recovery.

The analysis of aristolochic acid A in the herbal plants has recently developed by using high-performance liquid chromatography (HPLC). However, this method cost about 20 min. In this paper, the analytical method with pre-treatment procedure and system of ultra performance liquid chromatography, not

only removed most interfering peaks from the ultra performance liquid chromatography chromatogram, but also shortened the separation time.

EXPERIMENTAL

The samples analysized are dried root of aristolochiaceae. The samples were collected in Shimao, Xisuangbanna, Dehong and Honghe Prefecture, Yunnan Province, P.R. China. The samples were identified by Prof. Xi-Wen Li, Kunming Institue of Botany. For each sample, at least 0.5 kg of herbal samples were dried at room temperature for constant weight and pulverized to 80 mesh.

The analysis was performed on a Waters Acquity Ultra Performance LC equipped with quaternaey exchange pump, a multiple wavelength photodiode array detector, an autosampler and Empower chromatographic working station. A Waters Acquity BEH C_{18} column (1.7 μm, 2.1 mm × 100 mm) (Waters, USA) was utilized. Oasis MAX strong anion-exchange reversed-phase cartridge was used for sample preparation.

The aristolochic acid A standard (≥ 96 %) was purchased from Mark Bio-Technology Co. Ltd. (Tianjin , China). Acetonitrile (LC grade, Tedia), methanol (LC grade,Tedia) , Watson's pure water (Beijing, China), Other reagents were of analytical grade, including formic acid and sodium hydroxide.

The mobile phase composed of 0.1 % formic acid-acetonitrile (A) and 0.1 % formic acid-water (B). The gradient elution was programmed as follows: 0-4.50 min, 35-65 %A; 4.50-5.50 min, 65-98 % A; 5.50-5.51 min, 98 -35 % A; run time: 7.0 min; column tempeture, 35 ºC; flow rate: 0.3 mL/min; UV scan 210-410 nm (Fig. 1); detection wavelenfth: 322 nm; injection volume: 2 μL.

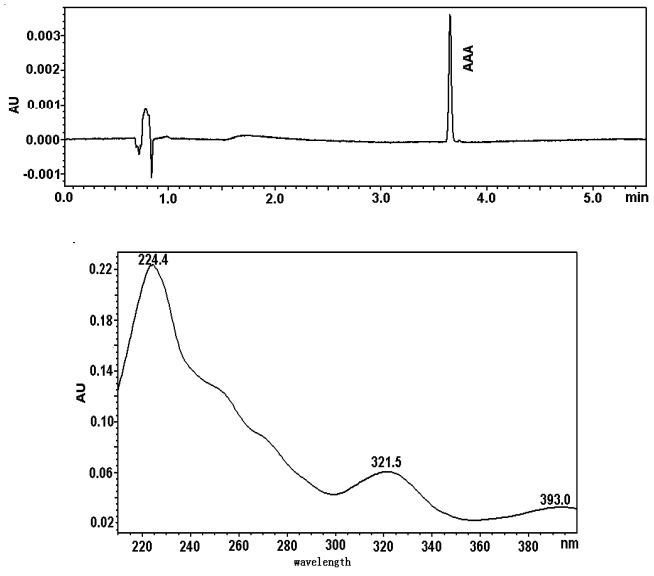

Fig. 1. Ultra performance liquid chromatography chromatogram and absorption spectra of aristolochic acid A

Preparation of standard solutions of aristolochic acid A: A stock standard solution aristolochic acid A (103 μg/mL) was prepared by dissolving 10.3 mg of aristolochic acid A in 100 mL methanol , kept at room temperature. A series of working solutions was prepared by diluting the stock standard solution.

Preparation of sample: The powdered sample (3 g) was extracted with 50 mL solution of 33 % methanol (containing 2 % formic acid). Ultrasonic extraction for 15 min and centrifugation at 4000 rpm for 10 min. The extracts were adjusted pH ≥ 10 by solution of 16 M sodium hydroxide. These samples can not analyzed directly by UPLC-PDA because the background peak is too high to cover the analyzing peak, so the following procedures are required: First, with 1 mL methanol, 1 mL pure water equilibrated anion solid-phase extraction cartridge (Oasis MAX Extraction cartridge). Second, loaded 1 mL extracts to carrier. Third, using 1 mL methanol, 1 mL methanol/2 % formic acid in water (6:4) to wash solid-phase, respectively. Finally, elution with 4 mL 5 % formic acid in water/acetonitrile (1:9), the eluate was filled up to 4 mL with acetonitrile and this was used as the sample solution for UPLC-PDA (Fig. 2).

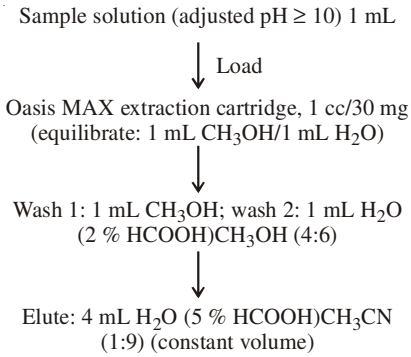

Fig. 2. Sample preparation with anion solid-phase cartridge for ultra performance liquid chromatography

RESULTS AND DISCUSSION

Solid-phase analysis of aristolochic acid A: The powdered sample was extracted with solution of 33 % methanol (containing 2 % formic acid). The main reasons are the following: For one thing, adding water in the extraction is contribute to samples homogenization. For another, acid substitution was extracted from sample when adding a certain amount of formic acid, comparison of different volume ratio of formic acid, it is found that 2 % formic acid is the most suitable.

In order to aristolochic acid ionized carboxylic anion with the quaternary ammonium ion anion-exchange cartridge make up to ionic bonds sufficiently, we adjust pH ≥ 10 (Fig. 3). Equilibrate ion anion-exchange cartridge with 1 mL methanol and 1 mL water, respectively. in order to matrix interference in sample has been cleaned, we tested different proportions of component solvent, have found that the aristolochic acid A was retained at cartridge when using 1 mL of methanol and 1 mL 2 % formic acid water:methanol (4:6) as washing liquid. The different proportions of component solvent as elution liquid were also tested. It is found that recovery rate above 91 % when using 5 % formic acid water/acetonitrile (1:9) eluted 4 mL.

Choice of chromatographic conditions: The aristolochic acid A is an organic acid and have a certain degree of solubility in water, have a poor peak shape when using reversed-phase

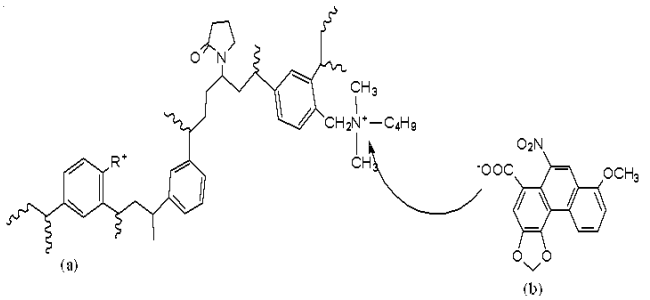

Fig. 3. Retention mechanism of aristolochic acid A on anion-exchange solid-phase extraction cartridge (a) the component of strong anion-exchange material (b) the anion of aristolochic acid A

chromatographic separated without adding acid. The mobile phase adding a certain amount of formic acid not only can inhibit the dissociation of aristolochic acid A, but that is beneficial to eliminated peak tailing. Thus need to adjust the mobile phase pH. Experimental results show that, 0.1 % formic acid was added in acetonitrile and water, respectively, when fixed acetonitrile-water ratio, the reproducibility is better.

Working curve: Standard stock solution is diluted gradually and injected 2 μL to UPLC, the standard solutions of aristolochic acid A different concentrations (C)'s peak area was measured, the results show that, the aristolochic acid A at 32.27-1236 ng/mL with peak area (A) have a good linear relationship, regression equation: A = 10.859C - 59.254, (r = 0.9999). Standard solution of small concentration of progressively diluted, when the signal to noise ratio S/N = 3, the concentration of the corresponding standard solutions to determine the detection limit. The detection limit was 1.65 ng/mL.

Analysis of aristolochic acid A in the samples: With pre-treatment processing approach treated and using UPLC analyzed *Aristolochia contorta Bge*, *Aristolochia fangchi*, *Aristolochia debilis sieb. Et Zucc* and *Asarum heterotropoides*, have found that *Aristolochia contorta Bge*, *Aristolochia fangchi* and the standard of aristolochic acid A have the same retention time and can isolated with interfering peaks (Fig. 4). While at the same retention time *Aristolochia debilis sieb. Et Zucc* and *Asarum heterotropoides* do not show any chromatographic peaks. The reason may be concentration of aristolochic acid A in the *Aristolochia debilis sieb.Et Zucc* and *Asarum heterotropoides* are so small than can not be detected (Table-1).

TABLE-1
AMOUNT OF ARISTOLOCHIC ACID A IN IN HERBAL PLANTS

Botanical name	Place of origin	Amount (%)
Aristolochia contorta Bge	Yunnan Province, Shimao	0.0432
Aristolochia fangchi	Yunnan Province, Xisuangbanna	0.0742
Aristolochia debilis sieb. Et Zucc	Yunnan Province, Dehong	N.D.
Asarum heterotropoides	Yunnan Province, Honghe	N.D.

N.D.: Mean that the content of aristolochic acid A is lower than the detection limit.

Method recovery and precision: The recovery test was carried out by adding aristolochic acid A to the samples (three different concentrations of markers: 0.1, 0.5 and 2.0 mg). The sample was prepared as above "preparation of sample" procedure

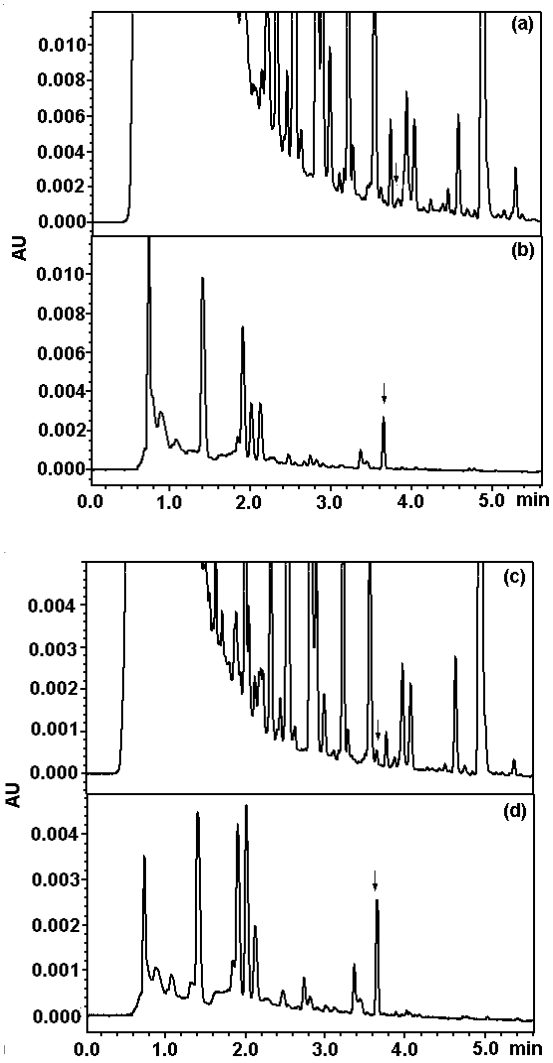

Fig. 4. Chromatograms of *Aristolochia contorta Bge* and *Aristolochia fangchi* (a) a crude extract of *Aristolochia contorta Bge*, (b) The sample of *Aristolochia contorta Bge* (applied to Oasis MAX), (c) a crude extract of *Aristolochia fangchi* (d) the sample of *Aristolochia fangchi* (applied to Oasis MAX)

and injected for UPLC analysis to calculate the amount of the aristolochic acid A founded. The results shown that the recoveries (n = 5) were ranged from 91-102 %.

The measurements of intra- and inter-day variability (determination of the same samples for seven times) were utilized to determine the precision of the developed method. The results show that the relative standard derivation of overall intra-day variations were less than 2.0 % and the relative standard derivation of inter-day variations were less than 2.5 %.

Conclusion

In this work, with a strong anion-exchange reversed-phase extraction cartridge (Oasis MAX Extraction cartridge) extracted aristolochic acid A from herbal plants and determination of aristolochic acid A by ultra-high-pressure liquid chromatography diode array detection. A strong anion-exchange reversed-phase extraction cartridge can purified samples by isolated acidic substances and eliminated interfering peaks from samples, the method of pre-treatment processing is simple and the loss of aristolochic acid A is less. In conclusion, the

method presents satisfied reproducibility and recovery and the lower detection limit. So, the method of pre-treatment processing has practical value that can be used for analysis of the content of aristolochic acid A in the herbal plants. With ultra performance liquid chromatography, injection volume is less, speed of analysis is rapid, sensitivity is high, improved the efficiency of the analysis.

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