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A Sensitive Enzyme-Linked Immunosorbent Assay for Detecting Carcinogenic Aristolochic Acid in Herbal Remedies

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Aristolochic acid, a naturally occurring nephrotoxin and rodent carcinogen, has been associated with the development of various nephropathies in humans. Developing a sensitive and rapid method to screen the aristolochic acid levels in herbal remedies is urgent for protecting public health. Polyclonal antibodies for aristolochic acid were generated from rabbits after the animals had been immunized with either aristolochic acid−ovalbumin (OVA) or aristolochic acid−keyhole limpet hemocyanin (KLH). A competitive indirect enzyme-linked immunosorbent assay (ciELISA) and a competitive direct enzyme-linked immunosorbent assay (cdELISA) were used for the characterization of the antibodies and for analysis of aristolochic acid contaminated in herbal medicine and diet pills. The antibody titers in the serum of rabbits immunized with aristolochic acid−OVA were considerably higher than those from aristolochic acid−KLH-immunized rabbits. The antibodies from the aristolochic acid−OVA-immunized rabbits were further characterized. In the ciELISA with aristolochic acid−KLH as the plate-coating antigen, the concentrations of the aristolochic acid mixture, aristolochic acid I, and aristolochic acid II that caused 50% inhibition (IC_{50}) of binding of antibodies to aristolochic acid−KLH were found to be 1.2, 0.7, and 18 ng/mL, respectively. When 0.25−5 μg/g of standard aristolochic acid was spiked to ground lotus seeds and then extracted with 0.01 M phosphate-buffered saline, the recovery rate was found to be 86.5% in the ciELISA. Analysis of aristolochic acid in herbal medicine and diet pills with ciELISA showed that 10 of the 12 examined samples were contaminated at levels from 0.6 to 655 μg/g. The presence of aristolochic acid was also confirmed by the high-performance liquid chromatography method.

KEYWORDS: Aristolochic acid; herbal medicine; antibodies; ELISA

INTRODUCTION

Aristolochic acid, an active component of the extract derived from *Aristolochia* spp., is a mixture of structurally related nitrophenanthrene carboxylic acids, mainly aristolochic acid I and aristolochic acid II (**Figure 1**) (*1*). Aristolochic acid is widely found in commercialized herbal products, although it has been proved to be a powerful nephrotoxic and carcinogenic substance in animal models (*2*−*4*). Both aristolochic acid I and aristolochic acid II can be metabolically activated to form DNA adducts through the simple reduction of their nitro groups. DNA damaged by aristolochic acid is not only responsible for tumor development but also for a destructive fibrotic process in the kidney (*5*−*8*).

Aristolochic acid in herbal medicines has been associated with the development of a novel nephropathy, designated aristolochic acid nephropathy (AAN), and even urothelial cancer in aristolochic acid patients (*9*−*11*). A unique type of rapidly

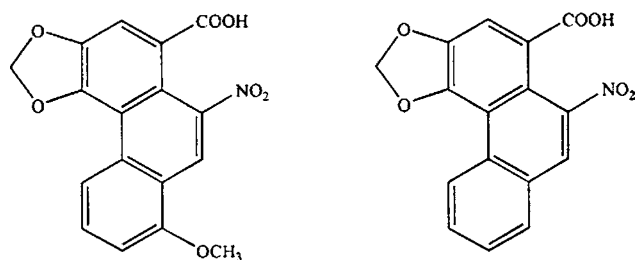

Figure 1. Chemical structures of aristolochic acid I and aristolochic acid II.

progressive renal failure resulting in end-stage renal disease has also been reported to occur in women taking a slimming regimen in Belgium in 1991 (*12*). These intoxications were found to be an accidental contamination of aristolochic acid originating from *Aristolochia fangchi* included in the diet pills. (*13*). Due to the potential public risks of ingestion of aristolochic acid, most European countries have banned its use in herbal medicines (*13*, *14*). The U.S. FDA also issued a Consumer Advisory to warn consumers away from dietary supplements and other botanic

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products containing aristolochic acid (*15*). However, many commercial products sold on the market were found to be contaminated with aristolochic acid, including confusion of close vernacular Chinese names or misuse in the drug preparations (*16*−*20*).

To help minimize the risk of human exposure to aristolochic acid, extensive research has been conducted to develop sensitive and specific methods for its detection in the herbal medicine. High-performance liquid chromatography (HPLC) and HPLC−mass spectrometry (HPLC-MS) with good accuracy and reproducibility are most widely employed for monitoring aristolochic acid (*17*, *18*, *20*−*22*). However, HPLC and HPLC-MS methods require highly qualified personnel and extensive sample cleanup as well as expensive equipment. Development of immunochemical approaches has led to more rapid and simple methods for monitoring and quantifying small molecule compounds such as mycotoxins and phycotoxins (*23*−*25*), but no enzyme-linked immunosorbent assay (ELISA) method for aristolochic acid analysis has been documented so far. In the present study, a method for the production of polyclonal antibodies against aristolochic acid was first developed, and a sensitive ELISA was also established to quantify the levels in various herbal medicines and diet pills.

MATERIALS AND METHODS

Materials. A mixture of aristolochic acid (29% of aristolochic acid I and 66% of aristolochic acid II) was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at −20 °C. Individual aristolochic acid I and aristolochic acid II were isolated from the mixture by an HPLC method (*22*). Bovine serum albumin (BSA), gelatin, ovalbumin (OVA), ammonium bicarbonate, Tween 20, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), 1,1′-carbonyldiimidazole, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were also obtained from Sigma. Goat anti-rabbit−peroxidase conjugate and keyhole limpet hemocyanin (KLH) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). HRP substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Neogen Corp. (Lexington, KY). Ammonium sulfate, absolute ethanol, and HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Microtiter plates and strips (low and high protein binding) were obtained from Nunc (Roskilde, Demark). The ELx 50 ELISA washer was purchased from Bio-Tek Instruments (Winooski, VT), and the Vmax automatic ELISA reader was from Molecular Devices Co. (Menlo Park, CA). Seven-week-old, female New Zealand white rabbits were obtained from Deer-Ho farm (Taichung, Taiwan). Freund's complete adjuvant containing *Mycobacterium tuberculosis* (H37 Ra) and Freund's incomplete adjuvant were obtained from Gibco BRL (Grand Island, NY). The HPLC equipment consisted of a Beckman System Gold 126 solvent module and a System Gold 168 detector (Fullerton, CA). The 0.45 μm syringe filter was obtained from Gelman Science (Ann Arbor, MI). All other chemicals and organic solvents used were of reagent grade or better.

Preparation of Various Aristolochic Acid Conjugates. *Conjugation of Aristolochic Acid to KLH or OVA.* Aristolochic acid was conjugated to KLH or OVA in the presence of a water-soluble carbodiimide, EDC, and NHS under the following conditions (*23*, *27*). In a typical experiment, EDC solution (1.5 mg of EDC in 0.1 mL of DMSO) and NHS solution (1.0 mg of NHS in 0.05 mL of DMSO) were freshly prepared and added to 2 mg of aristolochic acid mixture in 0.4 mL of DMSO. The reaction was kept at room temperature for 30 min. The mixture was added slowly to 4 mg of either KLH or OVA, which was dissolved in 0.4 mL of distilled water. The reaction was carried out at room temperature for 2 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15 M NaCl (PBS, pH 7.5) for 48 h with two exchanges of buffer and then lyophilized.

Preparation of Aristolochic Acid−Peroxidase. Conjugation of aristolochic acid to peroxidase was achieved by 1,1′-carbonyldiimidazole (CDI) under the following conditions (*28*). In a typical experiment, CDI solution (1 mg of CDI in 0.1 mL of acetone) was freshly prepared and added to 1 mg of aristolochic acid solution in 0.3 mL of acetone. The reaction was kept at room temperature for 30 min. The mixture was added slowly to 2.5 mg of HRP, which was dissolved in 0.5 mL of 0.1 M pH 9.6 carbonate buffer. The reaction was carried out at room temperature for 2 h. Then the reacted mixture was dialyzed against 2 L of 0.01 M PBS for 48 h with two exchanges of this buffer.

Production of Polyclonal Antibody. The schedule and methods of immunization were the same as those described previously (*23*). Two immunogens, aristolochic acid−OVA and aristolochic acid−KLH, were tested in four rabbits, with two rabbits for each immunogen. Each rabbit was injected intradermally at multiple sites on the shaved back (20−30 sites) with 500 μg of the immunogen in 1 mL of 0.01 M PBS mixed with 1 mL of Freund's complete adjuvant. For booster injections, the same amount of immunogen in PBS solution was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously at four sites on the thigh of each rabbit at the 5th and 12th weeks. Antisera were collected from the ears of the rabbits from the 5th week after the initial injection. The antisera were subjected to sequential steps of ammonium sulfate precipitation. The final precipitate in 35% ammonia sulfate was redissolved in distilled water equal to half of the original serum volume and then dialyzed against 2 L of PBS for 72 h at 4 °C with two changes of buffer.

Monitoring of Antibody Titers by Indirect ELISA (iELISA). The protocol for the iELISA was similar to that described previously (*26*). In general, 0.1 mL of aristolochic acid−KLH conjugate (1 μg/mL in 0.01 M PBS, pH 7.5) was added to each well of microtiter plate and kept at 4 °C overnight. After the plate had been washed four times with Tween−PBS (0.35 mL per well; 0.05% Tween 20 in 0.01 M PBS) using an automated ELISA washer, 0.17 mL of gelatin−PBS (0.17 mL per well; 0.1% gelatin in 0.01 M PBS) was added and allowed to incubate at 37 °C for 30 min. Then the plate was washed four times as described above, and 0.1 mL of diluted anti-aristolochic acid antiserum was added. After incubation at 37 °C for 1 h and washing with Tween−PBS, 0.1 mL of goat anti-rabbit IgG−HRP conjugate (1:20000 dilution) was added and incubated at 37 °C for 45 min. The plate was washed four times with Tween−PBS again, and 0.1 mL of TMB substrate solution (1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 per liter of potassium citrate buffer, pH 3.9) was added. After 10 min of incubation at room temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

Competitive Indirect ELISA (ciELISA). The protocol for ciELISA was the same as the above iELISA except that the step of diluted anti-aristolochic acid antiserum (0.1 mL) was replaced with 0.05 mL of anti-aristolochic acid antiserum and 0.05 mL of aristolochic acid standard/samples. The aristolochic acid standard with concentrations from 0.01 to 1000 ng/mL or extracted samples (0.05 mL per well in 0.01 M PBS) were added to each well, and then the anti-aristolochic acid−OVA antiserum (1:1000 dilution, 20 ng/mL in 0.01M PBS, 0.05 mL per well) was added to all wells and incubated at 37 °C for 50 min. After incubation, the plate was washed four times with Tween−PBS, and 0.1 mL of goat anti-rabbit IgG−HRP conjugate (1:20000 dilution) was added and incubated at 37 °C for 45 min. The plate was washed four times with Tween−PBS again, and 0.1 mL of TMB substrate solution was added. After 10 min of incubation at room temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

Competitive Direct ELISA (cdELISA). The protocol for cdELISA was essentially the same as previously described (*26*). The antibody collected from the 21st week was diluted in 0.01 M PBS, pH 7.5 (1:1000 dilution, 10 μg/mL), and 0.1 mL of the diluted form was coated onto each well. After the plate had been incubated at 4 °C overnight, it was washed with Tween−PBS followed by blocking with BSA−PBS (0.17 mL per well; 0.1% BSA in 0.01 M PBS) at 37 °C for 30 min. The plate was washed again with PBS−Tween four times, and then aristolochic acid standard (0.05 mL per well in 0.01 M PBS) concentrations from 0.01 to 1000 ng/mL or samples were added to each well, then aristolochic acid−HRP conjugate (1:500 dilution, 50

ng/mL in 0.01 M PBS, 0.05 mL per well) was added to all wells and incubated at 37 °C for 50 min. The plate was washed four times with Tween−PBS, and 0.1 mL of TMB substrate solution was added. After incubation at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. The absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

Recovery of Aristolochic Acid Added to Lotus Seed (*Nelumbinis semen*) Samples by ciELISA. A recovery study was carried out to test the efficacy of ciELISA for the analysis of aristolochic acid in lotus seed samples. One gram of ground lotus seed shown to be aristolochic acid-free by ELISA was spiked with aristolochic acid at concentrations ranging from 0.25 to 5 μg/g. A control sample with no toxin added was used as the blank. After storage at 4 °C for 1 day, each of the samples was extracted with an ultrasonic extractor using 0.01 M PBS twice (30 mL of PBS for 30 min and 20 mL for another 15 min), and the combined extracts were centrifuged at 7000 rpm for 5 min. The supernatant was filtered (0.45 mm pore size) and kept at −20 °C for ELISA and HPLC. At least two separate extracts were taken for each sample, and analysis on each extract was determined in triplicate.

ciELISA of Herbal Samples and Diet Pills Containing Aristolochic Acid. Six herbal medicine samples and six diet pills (in pill or powder form) purchased from local food stores in Taiwan were used to determine their levels. Briefly, each ground sample (1 g) was extracted with an ultrasonic extractor using 0.01 M PBS twice (30 mL of PBS for 30 min and 20 mL for another 15 min), and the combined extracts were centrifuged at 7000 rpm for 5 min. The supernatant was filtered (0.45 mm pore size) and kept at −20 °C for ELISA and HPLC. At least two separate extracts were taken for each sample, and analysis on each extract was determined in triplicate.

HPLC Analysis of Aristolochic Acid. Standard aristolochic acid and herbal samples containing aristolochic acid were subjected to HPLC analysis according to the procedure of Ioset et al. (*22*). The standard aristolochic acid and the sample extracts obtained as described above were passed through a low protein binding 0.45 μM filter (Gelman Science) prior to HPLC. A 25 cm × 4.6 mm, 5 μm, Lichrospher C18 reverse phase column (Merck) in conjunction with a 4 cm × 4.0 mm, 5 μm, Lichrospher C18 guard column (Merck) was equilibrated with a mobile phase consisting of 60% methanol in 0.05% TFA/water at a flow rate of 1 mL/min. After the sample was injected, aristolochic acid was eluted from the column with a linear gradient of methanol in 0.05% TFA from 60 to 100% methanol in 20 min. The chromatograms were monitored at 254 nm, and the absorbance data were analyzed with Beckman System Gold Nouveau software. A calibration curve was generated using aristolochic acid standards of 0.5, 1.0, 2.5, 5.0, and 10 μg/mL (R^2 = 0.99); each injection was 20 μL. The lowest detectable standard was 0.5 μg/mL, which corresponded to three times the standard deviation of the signal from the instrument.

RESULTS

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with aristolochic acid−OVA or with aristolochic acid−KLH were subjected to the indirect ELISA (iELISA). Typical titration curves of antibody titers over a period of 21 weeks were obtained from an aristolochic acid−OVA-immunized rabbit (**Figure 2**). Antibodies against aristolochic acid were detected in the sera of rabbits as early as 8 weeks after initial immunization. The antibody titer increased progressively with time, and the highest titer was found in the serum of rabbits at the 21st week after two subsequent immunizations. The antibody titers of the rabbits immunized with aristolochic acid−KLH were found to be considerably lower than those of the rabbits immunized with aristolochic acid−OVA (data not shown). Therefore, the 21st-week antiserum from the aristolochic acid−OVA-immunized rabbits was used in the subsequent studies.

Characterization of Antibodies. Both the ciELISA and cdELISA were used to determine the specificity of antibodies.

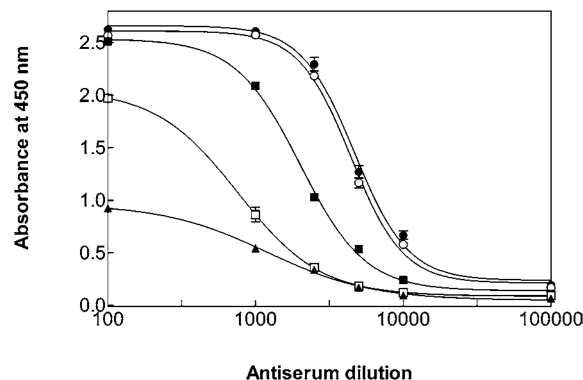

Figure 2. Determination of antibody titers for a representative rabbit after immunization with aristolochic acid–OVA by an aristolochic acid–KLH-based indirect ELISA. The antiserum was obtained 0 (▲), 8 (□), 12 (■), 18 (○), and 21 (●) weeks after immunization. All data were obtained from the average of three sets of experiments.

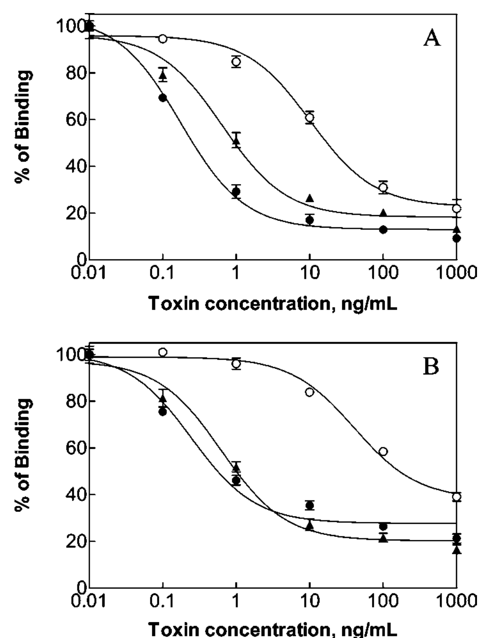

Figure 3. Cross-reactivity of anti-aristolochic acid antibodies with aristolochic acid I (●), aristolochic acid II (○), and aristolochic acid mixture (▲) as determined by (**A**) competitive indirect ELISA (ciELISA) [the absorbance of the control, A_0, with no toxin present, was 1.4] and (**B**) competitive direct ELISA (cdELISA) [the absorbance of the control, A_0, with no toxin present, was 1.6]. All data were obtained from the average of three sets of experiments.

In the ciELISA, aristolochic acid−KLH was coated to the wells of ELISA plates to serve as solid-phase antigen; the concentrations of aristolochic acid mixture, aristolochic acid I, and aristolochic acid II that caused 50% inhibition (IC_{50}) of binding of antibodies to the solid-phase aristolochic acid−KLH were found to be 1.2, 0.7, and 18 ng/mL, respectively (**Figure 3A**). When compared to the aristolochic acid mixture in the ciELISA, the relative cross-reactivities of the antibodies to aristolochic acid I and aristolochic acid II were calculated to be 171 and 6.7, respectively. Similar results were also obtained in the cdELISA. The IC_{50} values of aristolochic acid mixture, aristolochic acid I, and aristolochic acid II that blocked the binding of aristolochic acid−HRP to antibodies were 1.3, 1.0, and 258 ng/mL, respectively (**Figure 3B**). The relative cross-reactivities of the antibodies to aristolochic acid I and aristolochic acid II were found to be 130, and 0.5, respectively.

Table 1. Recovery Rate of Aristolochic Acid Spiked to Lotus Seed Samples by ciELISA

aristolochic acid[a] added (μg/g)	analytical recovery			
	μg/g	%	SD	CV%
0.25	0.23	92	0.03	13.0
0.5	0.45	90	0.05	11.1
1.0	0.78	78	0.06	7.6
5.0	4.3	86	0.12	2.7
overall		86.5		8.6

[a] Each toxin level had two samples, and each sample was analyzed in triplicate.

Table 2. ELISA and HPLC Analysis of Aristolochic Acid (AA) in Herbal Medicines and Diet Pills

sample		ELISA: AA and AA analogues (μg/g)	HPLC: AA I (μg/g)	HPLC: AA II (μg/g)
herbal medicines[a]				
1.	*Saussureae radix*	655	419	132
2.	*Aristolochiae caulis*	252	112	79
3.	*Asari herba cum radice*	2.9	ND[b]	2.2
4.	*Akebiae caulis*	3.8	ND	ND
5.	*Aristolochiae fangchi radix*	0.6	ND	ND
6.	*Aristolochiae fructus*	4.4	3.1	ND
diet pills[a]				
7.	pills	0.17	ND	ND
8.	pills	ND	ND	ND
9.	powder	ND	ND	ND
10.	powder	3.2	4.3	ND
11	powder	2.9	2.1	ND
12	powder	22	16.3	ND

[a] Each sample was extracted twice with PBS, and each extract was analyzed in triplicate. [b] Not detected.

Analytical Recovery of Aristolochic Acid Added to Lotus Seed Samples by ciELISA. To investigate the recovery rate of aristolochic acid in the ciELISA, the aristolochic acid mixture was first spiked into lotus seed samples at concentrations from 0.25 to 5 μg/g, and then the samples were extracted with 0.01 M PBS for recovery analysis. As shown in **Table 1**, the recovery rate for 0.25−5 μg/g of spiked aristolochic acid ranged from 78 to 92%, and the overall average was calculated to be 86.5% (CV, 8.6%).

Analysis of Aristolochic Acid in Herbal Medicine and Diet Pills with ciELISA. Six herbal samples and six diet pills were collected from local drug stores and subjected to ciELISA for aristolochic acid determination; the results are presented in **Table 2**. Ten of the 12 examined samples were found to be aristolochic acid positive, with levels that ranged from 0.17 to 655 μg/g. Sample 1 was found to have the highest level of aristolochic acid at 655 μg/g; sample 2 had aristolochic acid levels of 252 μg/g. The remaining four positive herbal samples contained aristolochic acid at <5 μg/g. Although two of the six diet pills were found to be aristolochic acid-free, the other four samples contained levels from 0.17 to 22 μg/g.

Confirmation of the Presence of Aristolochic Acid in Herbal Medicines and Diet Pills. To test the efficacy of the ciELISA for aristolochic acid determination in the herbal medicines and diet pills, the aristolochic acid standard and collected samples were further analyzed with the HPLC method; HPLC chromatograms are shown in **Figure 4**. Aristolochic acid I and aristolochic acid II standards were well identified with retention times of 11.4 and 9.5 min, respectively, under the linear gradient elution (**Figure 4A**). When the aqueous extracts of herbal medicine samples 1 and 2 were subjected to HPLC analysis, both chromatograms showed two peaks with retention times of 11.4 and 9.5 min expected for aristolochic acid I and aristolochic acid II, respectively (**Figure 4B**,**C**). Results of HPLC analysis of aristolochic acid in 12 examined samples are presented in **Table 2**. Both aristolochic acid I and aristolochic acid II were identified in two of the herbal medicines (samples 1 and 2). Sample 3 was found to contain 2.2 μg/g of aristolochic acid II. The remaining HPLC-positive samples contained only aristolochic acid I at levels ranging from 2.1 to 16.3 μg/g. Five samples, including two herbal medicines and three diet pills, were found to be free of aristolochic acid I and aristolochic acid II.

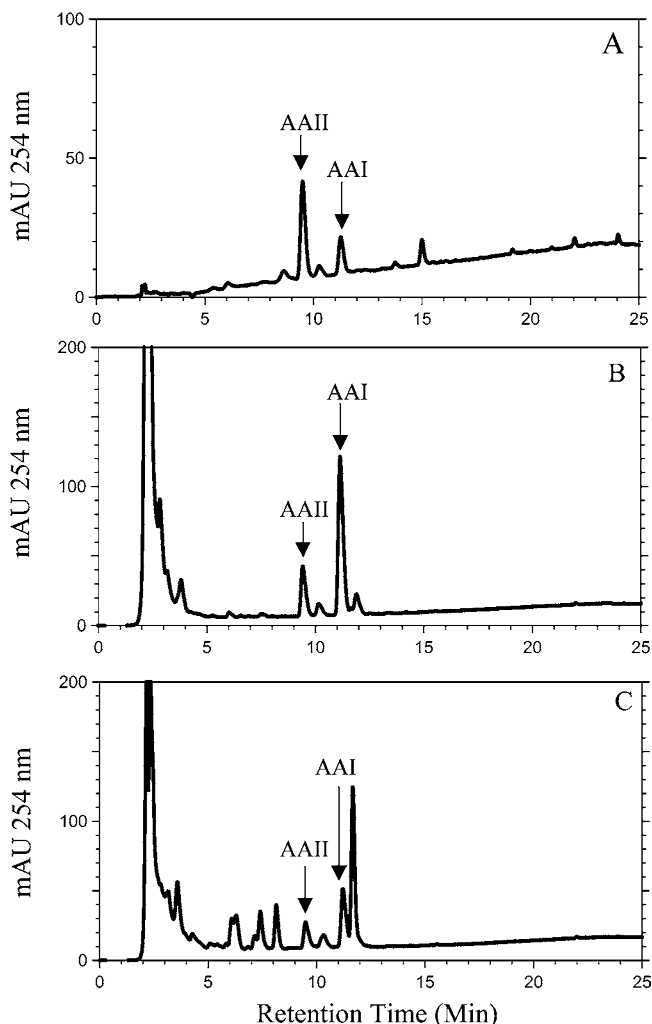

Figure 4. HPLC chromatograms of (**A**) standard aristolochic acid I (30 ng) and aristolochic acid II (70 ng), (**B**) herbal medicine sample 1 containing aristolochic acid I and II, and (**C**) herbal medicine sample 2 containing aristolochic acid I and II.

DISCUSSION

Aristolochic acid, a low molecular weight compound, is a group of nonimmunogenic toxicants similar to most mycotoxins and phycotoxins. To render it immunogenic, conjugating aristolochic acid to a protein carrier is necessary. For the production of specific antibodies against aristolochic acid, it was first conjugated to OVA or KLH as immunogen by a combination of carbodiimide and NHS. It is known that carbodiimide catalyzes the formation of amide bonds between carboxylic groups and amines, and NHS often assists carbodi-

imide coupling (*27*). Antibodies against both aristolochic acid−OVA and aristolochic acid−KLH were successfully generated and applied for developing highly sensitive immunoassays, including ciELISA and cdELISA. The cdELISA is generally known to be more rapid and sensitive and less time-consuming than ciELISA (*23*−*26*). However, when aristolochic acid−HRP served as the marker enzyme in the cdELISA for sample analysis, the aqueous extracts of herbal medicines showed a strong matrix interference, reducing the accuracy of cdELISA system. Therefore, in the present study ciELISA was chosen for further antibody characterization and sample analysis.

In the ciELISA, antibodies generated from the aristolochic acid−OVA-immunized rabbits were used, because they had an IC_{50} value 1 order lower than those from aristolochic acid−KLH-immunized ones. In the absence of matrix interference, the detection limit of aristolochic acid in the ciELISA, based on 10% of inhibition of binding of antibodies to the solid-phase aristolochic acid−KLH conjugate, was found to be 0.1 ng/mL (**Figure 3A**). Results from the recovery studies of aristolochic acid in lotus seeds showed that good recoveries were obtained at levels of 250 ng/g, based on 50-fold dilution, suggesting that the detection limit of the present method is close to 5 ng/g. The concentrations of aristolochic acid mixture, aristolochic acid I, and aristolochic acid II causing 50% inhibition of binding antibodies to the solid-phase aristolochic acid−KLH were 1.2, 0.7, and 18 ng/mL, respectively, suggesting that our antibodies showed similar affinities to aristolochic acid mixture and aristolochic acid I and less affinity to aristolochic acid II. The only difference between the chemical structures of aristolochic acid I and aristolochic acid II is the presence of a methoxy group on the structure (**Figure 1**), and it implies that the methoxy group could be a major recognition site of our antibodies.

When the developed ciELISA was applied to the analysis of herbal medicines and diet pills, 10 of 12 analyzed preparations were found to be aristolochic acid positive, and the levels ranged from 0.6 to 655 μg/g. Aristolochic acid II is a possible coexistent with aristolochic acid I in some samples, but the levels seem to be lower than those of aristolochic acid I in most of the examined samples. The results of HPLC analysis were consistent with ELISA data except that the extracts of samples 4, 5, and 7 showed neither aristolochic acid I nor aristolochic acid II peaks in the HPLC chromatograms. The aristolochic acid levels in samples 5 and 7 may be too low to be detected due to the detection limitation of HPLC, but the discrepancy in sample 4 could be caused by some unknown interferences in our ciELISA system. These data were similar to those of Lee et al. (*17*), who used the HPLC method to identify the aristolochic acid mixture in diet pills and found that the levels ranged from 22 to 694 ppm in 12 of 16 samples. Ioset et al. (*22*) also documented that 6 of 42 Chinese herbal samples obtained from the Swiss market were aristolochic acid positive using HPLC analysis, but the aristolochic acid levels were not reported. Although the adoption of aristolochic acid-generating herbs as regimen has been widely banned in many countries, inadvertent substitution of safe botanical products with aristolochic acid-generating species due to the confusion of their vernacular names and appearance is the major cause of aristolochic acid contamination in the examined herbal medicines (*10*, *14*, *15*, *19*, *21*).

There is an increased interest in alternative medicine, so consumers may choose various drug treatments or herbal health supplements to combat obesity and various diseases. However, before release onto the market, herbal medicine is not subjected to the same stringent scrutiny and controls as common drugs. Recently, more cases of urothelial carcinoma, related to Chinese herb nephropathy, have been reported in Belgium, France, Spain, Japan, Taiwan, and the United Kingdom (*10*, *13*, *14*, *19*, *21*, *29*); the presence of aristolochic acid and its derivatives in the plant preparations may play a causal role in these cases. To minimize the potential health risk of aristolochic acid to humans, a simple and rapid method will be needed for screening a large number of herbal samples. The sensitive and effective ciELISA first developed in the present study will be a powerful tool to evaluate the existence of aristolochic acid in herbal-related products.

ABBREVIATIONS USED

BSA, bovine serum albumin; CDI, 1,1′-carbonyldiimidazole; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; cdELISA, competitive direct ELISA; ciELISA, competitive indirect ELISA; iELISA, indirect ELISA; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; TMB, 3,3′,5,5′-tetramethylbenzidine.

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