

# Analysis of Aristolochic Acid I and II in Kampo Medicine Preparations

Takako Seto,\* Tomoko Hamano, Hiroko Shioda, and Hisashi Kamimura

Tokyo Metropolitan Research Laboratory of Public Health, 24–1 Hyakunin-cho 3-chome Shinjuku-ku, Tokyo 169–0073, Japan

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Herein reported are the detection methods of Aristolochic acid I (AA-I) and aristolochic acid II (AA-II), nephrotoxic components of the *Aristolochia* plants in *Aristolochiaceae*, in the Kampo medicine Toki-shigyaku-ka-goshuyu-shokyo-to. The confirmation of AA-I and AA-II was possible by the detection methods using a thin layer chromatography and a high performance liquid chromatography equipped with a Photodiode array detector (LC-PDA) and an electric ionization mass spectrometry detector. With the LC-PDA, it was possible to quickly detect AA-I and AA-II in the ten kinds of Kampo medicines in which the *Aristolochia fangchi* root or *Aristolochia mandshuriensis* stem may be misused. By using AA-I and AA-II purified from a commercial mixture by a preparative HPLC as reference standards, quantitative analyses of AA-I and AA-II were successfully carried out by use of the LC-PDA.

**Key words** — aristolochic acid, Kampo medicine, *Aristolochia*, liquid chromatography equipped with an electric ionization mass spectrometry detector, liquid chromatography equipped with a Photodiode array detector, herbal medicine

## INTRODUCTION

In Belgium and Japan, frequent occurrence of renal disorders called Chinese herbs nephropathy, the cause of which was discovered to be the presence of aristolochic acids in medicines or health foods made from crude drugs, have been reported.<sup>1,2)</sup> Aristolochic acid I (AA-I) acid II (AA-II) are components from the *Aristolochia* plants of *Aristolochiaceae*, and have been also discovered to cause mutagenicity and carcinogenicity.<sup>2)</sup> The five herbal medicines originating from the *Aristolochia* plants, *Aristolochia fangchi* (root), *Aristolochia mandshuriensis* (stem), *Aristolochia debilis* (radix, herb and fruit), *Aristolochia contorta* (fruit) and *Aristolochia contorta* (herb), are prescribed in Chinese Pharmacopoeia, and therefore are used in China. In Japan, herbal medicines originating from the *Aristolochia* plant are not permitted, even as raw materials in pharmaceutical preparations. The herbal medicines used respectively as “Boi” or “Mokutu” in Japan and China are shown in Table 1. In the Phar-

macopoeia of Japan, the herbal medicine called “Boi” is derived from the stem and rhizome of *Sinomenium acutum* (Sinomenium Stem); “Mokutu” in Japan is derived from the stems of *Akebia quinata* and *Akebia trifoliata* (Akebia Stem). Chinese Pharmacopoeia prescribes the root of *Stephania tetrandra* as “Boi” (Hun-boi); the stem of *Sinomenium acutum* is called Seihuto, and it is not used for “Boi” in China. The *Aristolochia fangchi* root is also used as the herbal medicine “Boi,” especially in southern China. On the other hand, Akebia Stem is not distributed in the Chinese market because there is no use for it; however, the *Aristolochia mandshuriensis* stem, *Clematis armandii* stem and *Clematis montana* stem are commonly used as “Mokutu.” Therefore, the *Aristolochia fangchi* root and *Aristolochia mandshuriensis* stem may be substituted for the Japanese herbal medicine Akebia Stem or Sinomenium Stem in Chinese-produced pharmaceutical preparations and health foods. We had a chance to analyze the Kampo medicine formulation, Toki-shigyaku-ka-goshuyu-shokyo-to, which caused the renal disorders in 1999. It was doubtful that the *Aristolochia mandshuriensis* stem was used in the formulation; therefore, we studied detection methods of aristolochic acids by employing HPLC attached with a Photodiode array detector (LC-PDA) and an electric ionization mass spectrometry detector (LC-

\*To whom correspondence should be addressed: Tokyo Metropolitan Research Laboratory of Public Health, 24–1 Hyakunin-cho 3-chome Shinjuku-ku, Tokyo 169–0073, Japan. Tel.: +81-3-3363-3231 ex. 3601; Fax.: +81-3-3368-4060; E-mail: tseto@tokyo-eiken.go.jp

**Table 1.** Definitions of Herbal Medicines used as “Mokutu” or “Boi” by the Pharmacopoeia of Japan and China

	“Mokutu”		“Boi”	
	proper name	origin	proper name	origin
Japanese Herbal Medicine	Akebia Stem	Stem of <i>Akebia quinata</i> Decne. and <i>Akebia trifoliata</i> Koidz. ( <i>Akebiaceae</i> )	Sinomenium Stem	Stem and Rhizome of <i>Sinomenium acutum</i> Rheder et Wilson ( <i>Menispermaceae</i> ) (= Seihuto of China)
Chinese herbal Medicine	Guanmutong	Stem of <i>Aristolochia manshuriensis</i> Kom. ( <i>Aristolochiaceae</i> )	Fangji (Fenfangji)	Root of <i>Stephania tetrandra</i> S. Moore ( <i>Menispermaceae</i> )
	Chuanmutong	Stem of <i>Clematis armandii</i> Franch. and <i>Clematis montana</i> Buch. ( <i>Ranunculaceae</i> )	Guangfangji	Root of <i>Aristolochia fangchi</i> Wu ( <i>Aristolochiaceae</i> )

EIMS) together with TLC. We also used the LC-PDA to detect AA-I and AA-II in nine other Kampo medicines containing Akebia Stem or Sinomenium Stem.

## MATERIALS AND METHODS

**Experimental Material** — Chemicals: Aristolochic acid (mixed reagents of AA-I and AA-II) was purchased from EXTRASYNTHESE S. A. (France) and BIOMOL RESEARCH LAB., Inc. (U.S.A.) Herbal medicines *Aristolochia fangchi* root and *Aristolochia mandshuriensis* stem were imported from China. The other herbal medicines for Kampo medicine model extracts are standardized articles by the Pharmacopoeia of Japan. Kampo medicine models: the prescribed herbal medicines (Table 2) for one day dose<sup>3)</sup> was decocted<sup>4)</sup> and double gauze filtered while it was still hot. The filtrate was freeze-dried to form an extract. Kampo medicine positive models contained the *Aristolochia* plant (Positive model): the *Aristolochia fangchi* root or the *Aristolochia mandshuriensis* stem were in place of Japanese crude drug Akebia Stem or Sinomenium, the others used were the same as used in the Kampo medicine models.

**Equipment** — Freeze-drying machine: Pilot plant freeze-drying machine Genesis 25EL type (BarTis Co., U.S.A.). Photodiode array-HPLC (LC-PDA): 880PU system (JASCO Co., Japan) and PDA detector 996 types (Waters Co., U.S.A.). Nuclear magnetic resonance spectrometry (NMR):  $\alpha$ -500 type (Japan Electron Optics Laboratory). Mass spectrometry-HPLC (LC-EIMS): Integrity system (Waters Co., U.S.A.)

**TLC Detection Method** — Sample preparation: a

50% MeOH extract of Kampo medicine extract or formulation (one day dose) was evaporated under reduced pressure, acidified with HCl, and extracted with Et<sub>2</sub>O. The Et<sub>2</sub>O layer was extracted with 3% NH<sub>4</sub>OH and acidified with 10% HCl. The NH<sub>4</sub>OH layer was extracted with Et<sub>2</sub>O and, after concentration the Et<sub>2</sub>O layer was tested with the TLC. TLC conditions: plate, silica gel 60F<sub>254</sub> plate; solvent system, CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (60 : 40 : 10); detection, UV irradiation and Dragendorff's reagent atomization.

**Liquid Chromatography Equipped with a Photodiode Array Detector Detection Method** — Sample preparation: 50 ml of 50% MeOH was added to a 1–0.5 day dose of Kampo medicine extract or formulation, then underwent ultra sonic irradiation for 30 min and next was filtrated (45  $\mu$ m pore size) to give a sample solution for HPLC analysis. Standard solution: Aristolochic acid (about 5.0 mg) was dissolved in the 50 ml MeOH. The LC-PDA conditions are shown in Table 3.

**Liquid Chromatography Equipped with an Electric Ionization Mass Spectrometry Detector Identification** — Sample preparation: The refined sample prepared as well as the TLC sample was suspended with 50% MeOH and loaded on MEGA Bond Elut C18. The aqueous eluent was used as an LC-EIMS sample solution. The LC-EIMS conditions are shown in Table 3.

**HPLC Quantitative Analysis** — Sample solution for HPLC: Kampo medicine extract or formulation about 1–0.5 day dose was accurately weighed and 50% MeOH 30 ml was added on it, and underwent ultra sonic irradiation (30 min). After centrifuging (3000 rpm, 5 min) and removal of the solvent, the residue was extracted twice with 50% MeOH (10 ml, 10 min). The supernatants were combined, made up

**Table 2.** Detection of Aristolochic Acid I and II in Kampo Medicines Used *Aristolochia* Herbal Medicines by HPLC-PDA

Kampo Medicine Formula	Prescribed Herbal Medicines	Substituted Herbal Medicine	AA-I	AA-II
Kami-gedoku-to	Coptis Rhizome, Phellodendron Bark, Bupleurum Root, Japanese Gentian, Tarcum Crystallium, Glycyrrhiza, Rhubarb, Scutellaria Root, Gardenia Fruit, Artemisia Capillaris Flower, Akebia Stem,* Cimicifuga Rhizome, Juncus Stem Medulla	<i>Aristolochia mandshuriensis</i> Stem	○	△
Gorin-san	Polia Sclerotium, Japanese Angelica Root, Scutellaria Root, Glycyrrhiza, Peony Root, Gardenia Fruit, Rehmannia Root, Alisma Rhizome, Akebia Stem,* Tarcum Crystallium, Plantago Herb	<i>Aristolochia mandshuriensis</i> Stem	○	○
Shohu-san	Japanese Angelica Root, Rehmannia Root, Gypsum, Saposhnikovia Root, Atractylodes Lancea Rhizome, Akebia Stem,* Arctii Fructus, Anemarrhena Rhizome, Sesame Seed, Cicadae periostracum, Sophora Root, Schizonepeta Spike, Glycyrrhiza	<i>Aristolochia mandshuriensis</i> Stem	○	○
Tsudo-san	Japanese Angelica Root, Rhubarb, Mirabilite, Immature Orange, Magnolia Bark, Citrus Unshiu Peel, Akebia Stem,* Safflower, Sappan Wood, Glycyrrhiza	<i>Aristolochia mandshuriensis</i> Stem	○	○
Toki-shigyaku-to	Japanese Angelica Root, Cinnamon Bark, Peony Root, Akebia Stem,* Jujube, Asiasarum Root, Glycyrrhiza	<i>Aristolochia mandshuriensis</i> Stem	○	○
Toki-shigyaku-kagoshuyu-shokyo-to	Japanese Angelica Root, Cinnamon Bark, Peony Root, Akebia Stem,* Jujube, Asiasarum Root, Glycyrrhiza, Evodia Fruit, Ginger	<i>Aristolochia mandshuriensis</i> Stem	○	○
Ryutan-shakan-to	Japanese Angelica Root, Rehmannia Root, Akebia Stem,* Scutellaria Root, Alisma Rhizome, Plantago Seed, Japanese Gentian, Gardenia Fruit, Glycyrrhiza	<i>Aristolochia mandshuriensis</i> Stem	○	○
Boi-ogi-to	Sinomenium Stem,** Astragalus Root, Atractylodes Lancea Rhizome, Ginger, Jujube, Glycyrrhiza	<i>Aristolochia fangchi</i> Root	○	○
Boi-bukuryo-to	Sinomenium Stem,** Astragalus Root, Cinnamon Bark, Polia Sclerotium, Glycyrrhiza	<i>Aristolochia fangchi</i> Root	○	○
Sokei-kakketsu-to	Japanese Angelica Root, Rehmannia Root, Cnidium Rhizome, Atractylodes Lancea Rhizome, Polia Sclerotium, Peach Kernel, Peony Root, Achyranthes Root, Clematis Root, Sinomenium Stem,** Notopterygium Rhizome, Saposhnikovia Root, Ginger, Citrus Unshiu Peel, Angelica Dahurica Root, Glycyrrhiza, Japanese Gentian	<i>Aristolochia fangchi</i> Root	○	×

\*: substituted for *Aristolochia mandshuriensis* Stem, \*\*: substituted for *Aristolochia fangchi* Root. Spectrum Match, ○: good, △: poor, ×: bad.

to 50 ml, and filtrated (45  $\mu$ m pore size) to give a sample solution. Standards: AA-I and AA-II were purified from a commercial mixture by the preparative HPLC, and identified by two-dimensional NMR. The HPLC conditions are shown in Table 3.

## RESULTS AND DISCUSSION

### Detection Methods of Aristolochic Acid I and Aristolochic Acid II

A lot of components are present in herbal drug

preparations such as Kampo medicines, and they can be observed on the HPLC chromatogram. Only mixtures of AA-I and AA-II are marketed. Trying to confirm elements without pure standards by identifying *R<sub>f</sub>* values of the TLC spots or retention time of HPLC peaks may be misleading. Actually, there are two reports<sup>2,5)</sup> in which AA-I and AA-II peaks of HPLC were reversed on the assignments. Though AA-I and AA-II are both purified by the preparative HPLC, it is desirable to quickly confirm their presence in mixed standard materials. Thus, we carried out TLC, LC-PDA and LC-EIMS analyses on a posi-

Table 3. HPLC Conditions

	Column	Column Temperature	Mobile Phase	Flow Rate ml/min	Detection
LC-PDA	TSKgel ODS-80T <sub>M</sub> (5 μm, 4.6 mm i.d. × 150 mm, TOSOH)	40°	Water-Acetonitrile- Phosphoric acid (595 : 405 : 1)	1	PDA 200–600 nm
Quantitative HPLC	TSKgel ODS-80T <sub>M</sub> (5 μm, 4.6 mm i.d. × 150 mm, TOSOH)	40°	Water-Acetonitrile- Phosphoric acid (595 : 405 : 1)	1	PDA 200–600 nm Detected at 254 nm
LC-EI(MS)	Nova-PakR <sup>®</sup> C18, 60Å (4 μm, 2 mm i.d. × 150 mm, Waters)	40°	Water-Acetonitrile (1 : 1)	0.2	Ionization Voltage, 70 eV Ion Sours Temperature, 200° Nebulizer Temperature, 70° Flow Rate of He, 0.2 l/min
Preparative HPLC	TSKgel ODS-80T <sub>M</sub> (10 μm, 2 Inch i.d. × 300 mm, TOSOH)	Room Temperature	Water-Acetonitrile- Phosphoric acid (600 : 400 : 1)	30	UV 254 nm

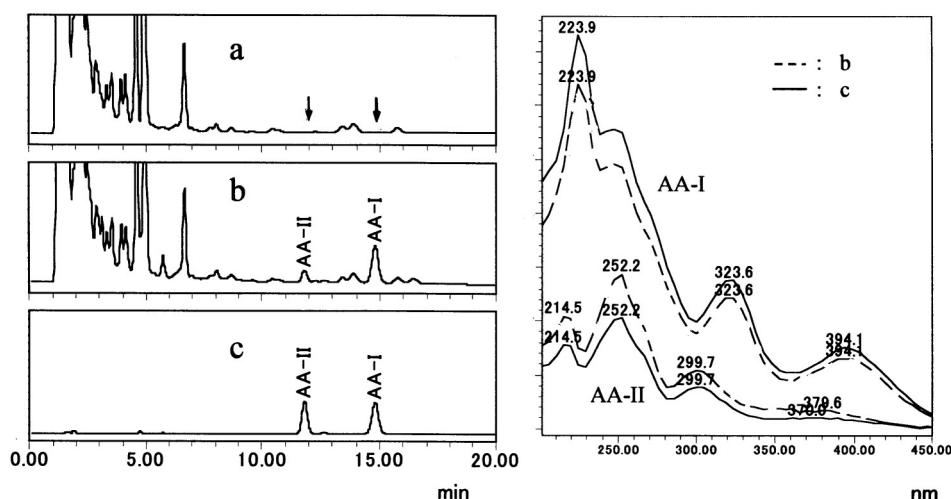


Fig. 1. LC-PDA Chromatograms of Kampo Medicine Toki-shigyaku-ka-goshuyu-shokyo-to and the Spectra of Their Aristolochic Acid I (AA-I) and Aristolochic Acid II (AA-II)

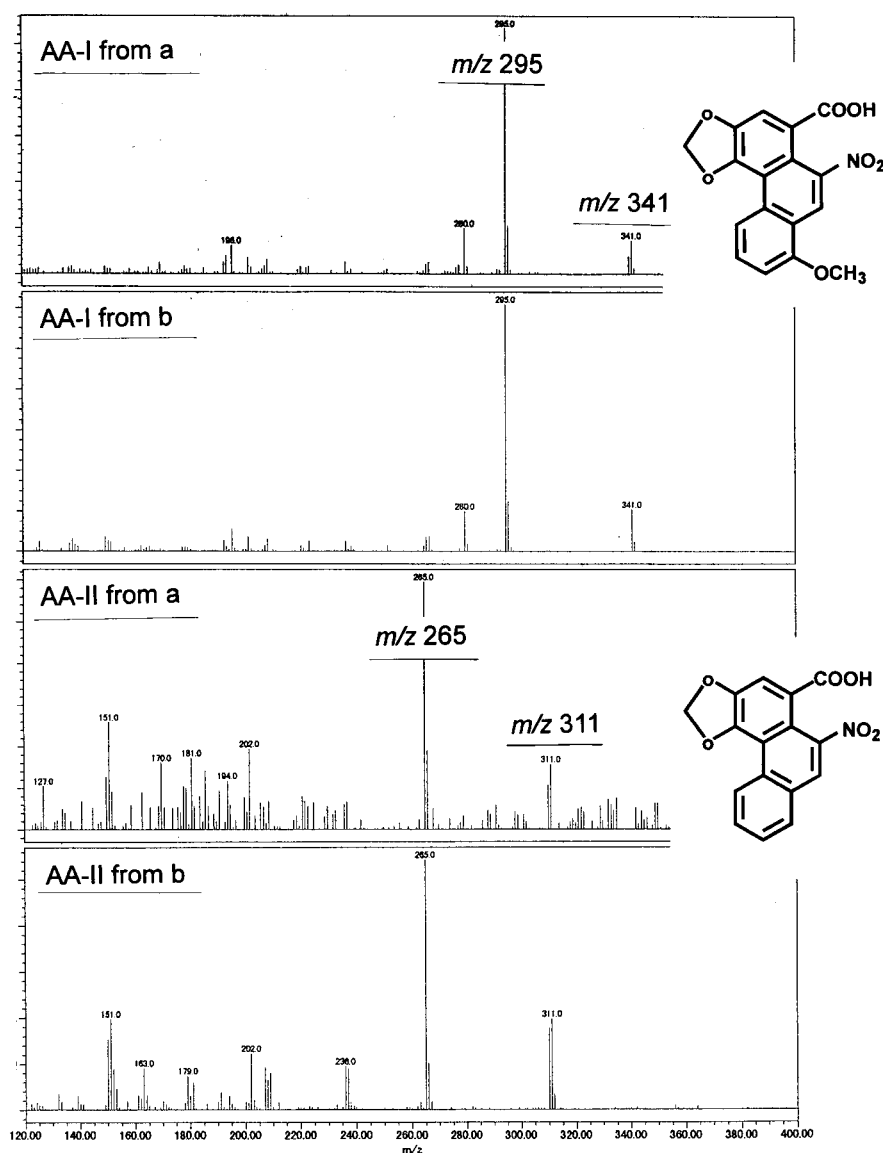
a: Akebia Stem was used. b: *Aristolochia mandshuriensis* stem was used (positive control). c: standard (mixture of AA-I and AA-II).

tive model of Toki-shigyaku-ka-goshuyu-shokyo-to.

AA-I and AA-II showed  $R_f$  values of 0.51 and 0.48 respectively, and their separation was excellent. However, distinguishing the two was difficult. Under UV 254 nm irradiation both showed up as spots of the same dark color. When sprayed with Dragendorff's reagent, they both were bitter orange brown in color. The spots of AA-I and AA-II were obtained from the positive model of Toki-shigyaku-ka-goshuyu-shokyo-to. This method was useful as one of the detection methods since it did not require the use of expensive equipment.

The LC-PDA results are shown in Fig. 1. The UV spectra of the AA-I peak and the AA-II peak are

obtained at 14.8 and 11.8 min respectively, and they had the maximum absorptions at 224, 324 and 394 nm and at 215, 252 and 300 nm respectively under the examined conditions. In literature, the maximum absorptions of the AA-I<sup>6)</sup> in ethanol solution were found to be 221 nm ( $\log \epsilon$  4.47), 250 (4.51), 317 (4.05) and 388 (3.78); and for AA-II<sup>7)</sup> they were 249 nm ( $\log \epsilon$  4.58), 296 (4.12) and 362 (3.59). These disagreements were thought to arise according to a difference of the matrix. Since AA-I and AA-II were respectively identified by not only the retention times but also by the spectra from the positive model of Toki-shigyaku-ka-goshuyu-shokyo-to, this method also proved to be effective. Absences of



**Fig. 2.** EIMS Spectra of Aristolochic Acid I (AA-I) and Aristolochic Acid II (AA-II) Peaks from LC-EIMS Chromatograms of Kampo Medicine Toki-shigyaku-ka-goshuyu-shokyo-to Used *Aristolochia Mandshuriensis* Stem and Standard

a: Kampo medicine Toki-shigyaku-ka-goshuyu-shokyo-to used *Aristolochia mandshuriensis* stem (positive control), b: standards.

AA-I and AA-II peaks were confirmed in the Kampo medicine model of Toki-shigyaku-ka-goshuyu-shokyo-to. As a result of the additional examination of the Kampo medicine model, the detection limit was 80 ppm.

The MS spectrum of LC-EIMS peaks of AA-I and AA-II gave molecular ion peaks,  $m/z$  341 and  $m/z$  311 respectively, and fragment peaks that formed by the elimination of the nitro group,  $m/z$  295 and  $m/z$  265, as base peaks, as shown in Fig. 2. These MS data, including the other fragments, agreed with a literature description.<sup>7)</sup> Usually acetic acid was added to the HPLC mobile phase in order to control ionization of testing materials. However, it was found

that the MS spectrum had changed greatly, making it difficult to compare it with literature data by use of acetic acid in the HPLC mobile phase. Therefore, although the addition of acetic acid improved the separation of the peaks, it was not used in this case. For the ion source protection, a positive model of Toki-shigyaku-ka-goshuyu-shokyo-to was analyzed after refinement by the liquid-liquid distribution method and then with an ODS cartridge. As a result, the HPLC peaks and the MS spectrum that corresponded to AA-I and AA-II could be detected from the positive model of Toki-shigyaku-ka-goshuyu-shokyo-to. LC-EIMS was useful for quick identification even when pure standards could not be ob-

tained. LC-EIMS is a comparatively new technique owing to EI of the ionization mode, and it enabled us to refer to the abundant MS spectrum data that had been described in literature or saved in libraries.

### Application of Aristolochic Acid I and Aristolochic Acid II Detection in Kampo Medicine

Among 210 kinds of the Kampo medicine admitted for non-prescription drugs by the Ministry of Health, Labor and Welfare in Japan, a total of ten kinds of Kampo medicines contain Akebia Stem (seven) or Sinomenium Stem (three) as ingredients, as are shown in Table 2. It is feared that their proper ingredients could be replaced by the *Aristolochia mandshuriensis* Stem or *Aristolochia fangchi* Stem. Therefore, the LC-PDA detection method for aristolochic acid was applied to these ten Kampo medicines, and the results along with their formulations, are shown in Table 2. The peak identified as AA-I by the UV spectrum was detected from all of their positive models, *i.e.* freeze-dried extracts of decoctions in which *Aristolochia mandshuriensis* Stem or *Aristolochia fangchi* Stem was substituted for Akebia Stem or Sinomenium Stem. A good match with the UV spectrum of the AA-II standard was not achieved for Sokei-kakketu-to and Kamigedoku-to because of the presence of other undetermined elements. In short, both AA-I and AA-II, or at least AA-I, could be detected in all of the medicines. In conclusion, if herbal medicines that come from the genus *Aristolochia* are used as Akebia Stem or Sinomenium Stem in these ten kinds of Kampo medicines, the injurious ingredient aristolochic acids can be simply and successfully detected using LC-PDA.

### Quantitative HPLC Analysis of Aristolochic Acid I and Aristolochic Acid II

Quantitative HPLC analysis of AA-I and AA-II was achieved by using reference standards refined from the commercial mixture by the preparative HPLC. These standards were single in TLC and HPLC, and NMR spectrum corresponded to literature values, respectively. As the solvent for extraction, 50% MeOH is excellent. The recoveries of added AA-I and AA-II were 98% and 97%, respectively.

In conclusions, the confirmation of AA-I and AA-II in the Chinese medicine formulation Tokishigyaku-ka-goshuyu-shokyo-to was possible by

using TLC, LC-PDA, and LC-EIMS detection methods. LC-PDA and LC-EIMS detection methods both were effective in distinguishing AA-I and AA-II peaks by their spectra. The LC-PDA detection method was suitable for quick detection of said acids. The LC-EIMS detection method was excellent in identifying HPLC peaks, since the MS (EI) data could be referred to the rich data. Use of the *Aristolochia* plants could be detected in all the extracts of ten kinds of Chinese medicine using the LC-PDA detection method. With LC-PDA and using AA-I and AA-II purified from a commercial mixture by a preparative HPLC as reference standards, quantitative analyses of AA-I and AA-II were successfully carried out.

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