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Short communication

Analysis of aristolochic acid in nine sources of Xixin, a traditional Chinese medicine, by liquid chromatography/atmospheric pressure chemical ionization/tandem mass spectrometry

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Abstract

Aristolochic acid I (AA-I), which is a known nephrotoxin, is found in a commonly used Chinese medicine, Xixin, that originates from nine *Asarum* species (*Aristolochiaceae*) found in China. A method has been developed using reversed-phase liquid chromatography coupled with atmospheric pressure chemical ionization (APCI) tandem mass spectrometry under the positive ion detection mode [LC/(+)APCI/MS/MS] to determine the amount of AA-I in Xixin. The limit of detection of AA-I, estimated by monitoring with LC/MS/MS, was at the low μg/l level. By applying this method to methanol extracts of nine *Asarum* species, the concentrations of AA-I were found to range from 3.3 ng/mg (*Asarum sieboldii*) to 3376.9 ng/mg (*Asarum crispulatum*).

Keywords: *Aristolochiaceae*; Aristolochic acid; *Asarum* species; LC/APCI/MS/MS; Xixin

1. Introduction

Aristolochic acid (AA) is a known nephrotoxin and potential carcinogen that is found in *Aristolochia* spp., *Bragantia* spp. and *Asarum* spp. In 1999, Hashimoto et al. [1] reported the quantitative analysis of AA-I and AA-II (Fig. 1) by HPLC/UV, the detection limit of which was 1 ppm, and found that AA-I and II were present in all the plants of the genus *Aristolochia*. In *Asarum* spp., however, only two (*Asarum himalaicum* and *Asarum splendens*) out of eight species were found to contain AA-I and, even then, only in trace amounts.

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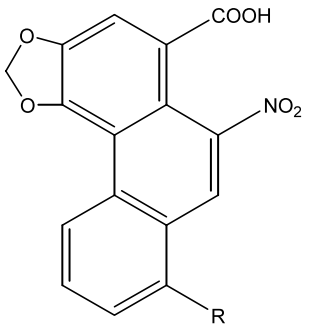

aristolochic acid I: R =OMe
aristolochic acid II; R =H

Fig. 1. Structures of AA-I and II.

"Xixin" (*Asari* radix, "Saishin" in Japanese) is one of the most important crude drugs in Chinese medicine and is exported all over the world. It has been used as an analgesic and antitussive, and has been studied for its active anti-allergy components [2]. Since Xixin was prepared originally from a few *Asarum* spp. (*Aristolochiaceae*) over the China and it is important that the AA content of Xixin remains under certain limits (less than 0.5 ppm in the market product), the analysis of AAs in their natural sources used for herbal medicine needs to be undertaken and their quantities declared.

A conventional reversed-phase HPLC survey of methanol extracts of Xixin gave a complicated chromatogram that, when viewed at its characteristic UV absorption at 380 nm, displays only a tiny peak (500 ppb) that corresponds to the spiked standard AA-I (Fig. 2). Thus, it appears difficult to analyze the trace amounts of AA-I in Xixin extracts by using a UV detector. Instead, applying LC/MS/MS overcomes this problem and it has become the method of choice for the analysis of traces of AAs.

In this paper, the application of LC/MS/MS with atmospheric pressure chemical ionization (APCI) under the positive ion detection mode is reported for the analysis of AA in Xixin. Recently, Simmonds published [3] an analysis of AA in herbal remedies in which LC/serial mass spectrometry was used. In contrast to that method, which uses a basic aqueous ammonium mobile phase, we chose a simple acidic aqueous phase (0.005% trifluroacetic acid (TFA)) to achieve about the same detection limits in general. By analyzing extracts of nine *Asarum* species, the effectiveness of this proposed method was tested by determining the levels of AA in actual samples.

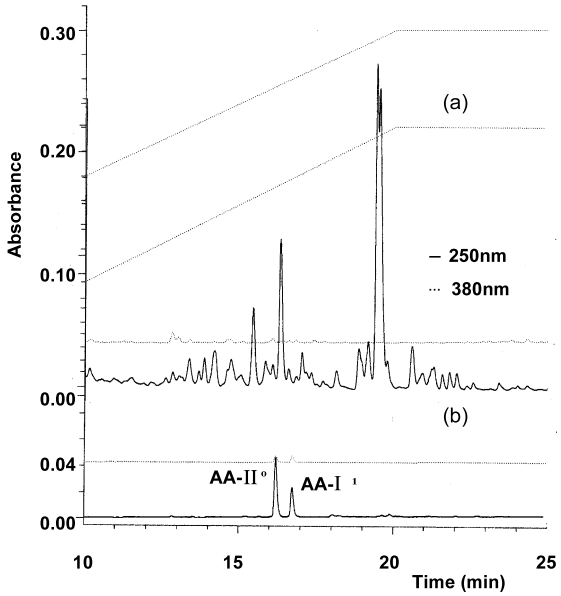

Fig. 2. Chromatograms (LC, UV detection) of (a) an extract of *A. sieboldii* (Xixin sample) and (b) a standard solution of AA-I (500 ppb) and AA-II (778 ppb). HPLC conditions: see Section 2.

2. Experimental

2.1. Plant material

Asarum species were collected in China and identified at Beijing University. The cold methanol extracts of nine *Asarum* species were weighed: *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag (16.9 mg); *Asarum crispulatum* C.Y. Cheng and C.S. Yang (3.9 mg); *Asarum forbesii* Maxim (5.9 mg); *A. himalaicum* Hooh. F. and Thoms. Ex Klotzsch (2.0 mg); *Asarum sieboldii* Miq (9.2 mg); *Asarum debile* Franch (10.0 mg); *Asarum maximum* Hemsl (20.0 mg); *Asarum ichangense* C.Y. Cheng and C.S. Yang (12.5 mg); *Asarum fukienense* C.Y. Cheng

and C.S. Yang (8.8 mg; 74.7 mg extracted with hot methanol).

2.2. Instrumentation

HPLC/UV analyzes were carried out on a Beckman HPLC equipped with a 125 pump and a photodiode array detector and running the Gold system. HPLC conditions: 20% (v/v) acetonitrile (containing 0.005% TFA, pH 3.42), increasing to 100% acetonitrile over 20 min, and then holding at 100% of acetonitrile for another 10 min.

HPLC/MS was performed on an LCQ™ instrument (Finnigan MAT, San Jose, CA) equipped with an APCI source, which was connected to a Surveyor LC pump. APCI was performed by setting the discharge current to 5 μA. The vaporizer and capillary temperatures were set at 525 and 180 °C, respectively. The sheath gas, which was nitrogen, had a flow rate of 50 units. The mass range for an APCI full scan was set from *m/z* 100 to 1000. Product-ion spectra were scanned from *m/z* 100 to 400. The precursor ions of AA-I and AA-II were selected at *m/z* 324 $[M+H-H_2O]^+$ and 326 $[M+15]^+$, respectively. For chromatographic separation, a Merck Lichrocart Purospher RP-18e (5 μm) column (125 mm × 4 mm i.d.) was used at ambient temperature and with a flow rate of 1 ml/min. The solvent system consisted of a step-gradient starting at 20% (v/v) methanol (containing 0.005% TFA) in water (0.005% TFA, pH 3.42), increasing to 100% MeOH over 15 min, holding at 100% MeOH for another 6 min, and then quickly returning to 20% MeOH over 2 min. The sample volume injected was 20 μl. Under these LC conditions, AA-I and II, and an internal standard, indomethacine, have retention times of 12.6, 11.9 and 13.0 min, respectively.

2.3. Preparation of standards and samples

A mixture of AA-I (38%) and AA-II (59%), purchased from Sigma (“lot.51K1547”), was used as the standard. A standard stock solution was prepared by dissolving 25 mg of AA-I and II in MeOH, transferring the solution to a 50-ml volumetric flask, and then adding MeOH to make the volume up to 50 ml. From this solution, a series of eight standards with concentrations of AA-I of 10, 25, 50, 100, 250, 500, 750 and 1000 ng/ml (with the corresponding concentrations of AA-II being 15.5, 38.8, 77.6, 155.3, 388.2, 776.3, 1164.5 and 1552.6 ng/ml) were prepared as calibration samples.

The MeOH extract of each of nine *Asarum* species was dissolved in 1 ml of MeOH, sonicated, and then filtered over a 0.45 μm filter to give an analytical sample for LC/MS. In a few cases, the sample was diluted into a further 3 or 5 ml of MeOH to allow detection in the range of the calibration curve.

2.4. Calibration curves

Indomethacine was used as an internal standard. The calibration curves were plotted as the ratio of the peak areas of analyte:internal standard versus the analyte concentration (ng/ml). The concentrations of the eight standards ranged from 10 to 1000 ng/ml for AA-I and from 37.2 to 1487.2 ng/ml for AA-II; the internal standard, indomethacine (1 μg/ml), was added to each solution. A weighted ($1/x^2$) linear regression line was fitted over the concentration range 10–1000 ng/ml. The concentration of AA-I in the nine samples of the *Asarum* species were calculated from the ratios of the peak areas and are expressed in units of ng per mg of the extract.

3. Results and discussion

For the LC/MS analysis, the best conditions for ionization of AA in the mass spectrometer were obtained using the APCI (+) ionization mode; using either the APCI (−) or ESI (±) ion modes gave poor ion count, as was also reported by Simmonds [3]. For the precursor ion, Simmonds chose $[M+NH_4]^+$, generated by adding 0.1% ammonium acetate (under basic conditions; i.e. pH 7.4) to the mobile phase or 0.1% acetic acid as aqueous phase with ammonia added post-column. We have found, however, that a great enhancement occurred (about four-times) in the ion count of $[M+H-H_2O]^+$ when 0.005% TFA was used in the aqueous mobile phase instead of 1% acetic

acid. Under these acidic conditions, the positive-ion APCI mass spectrum of AA-I (MW = 341) revealed that the ion at *m/z* 324 $[M+H-H_2O]^+$ had the greatest intensity, with a minor ion at *m/z* 294 $[M+H-H_2O-NO]^+$. For AA-II (MW = 311), three major ions were obtained at *m/z* 326 [M + 15], 294 $[M+H-H_2O]^+$, and 264 $[M+H-H_2O-NO]^+$, respectively (Fig. 3). The peak at *m/z* 326 is the base peak and is believed to be the methanol adduct ion of $[M+H-H_2O]^+$, which was supported by the tune experiment in which the signal's intensity increased by ten-times when the temperature of the capillary decreased from 250 to 180 °C; the ions at *m/z* 294 and 264 are similar to those obtained in the spectrum of AA-I.

Under MS/MS conditions, with optimized collision-induced dissociation (CID) at 30%, the precursor ion of AA-I at *m/z* 324 was observed to fragment almost exclusively to product ions at *m/z* 280 $[M+H-H_2O-CO_2]^+$ and 265 $[M+H-H_2O-CO_2-CH_3]^+$ (Fig. 3). The corresponding ion of AA-II at 294 $[M+H-H_2O]^+$ was not chosen as its precursor ion because of low efficiency of fragmentation; instead, the adduct ion of AA-II at *m/z* 326 $[M+H+CH_3OH-H_2O]^+$, which is the base peak of AA-II, was fragmented into product ions at *m/z* 294 $[M+H-H_2O]^+$, 295 $[M+H+CH_3OH-H_2O-CH_3O]^+$ and their corresponding ions due to the loss of CO at *m/z* 266 and 267, with CID at a 30% relative collision energy. The chromatograms were obtained over 15 min and displayed no other peaks for AA-I. High selectivity is a property of this analytical method.

Fig. 4 displays the selected reaction monitoring (SRM) chromatograms of (a) AA-I with an extracted-ion chromatogram at *m/z* 280 from the MS/MS scan of the precursor ion at *m/z* 324, and (b) AA-II with an extracted-ion chromatogram at *m/z* 294 ± 1 plus 266 ± 1 from the MS/MS scan of

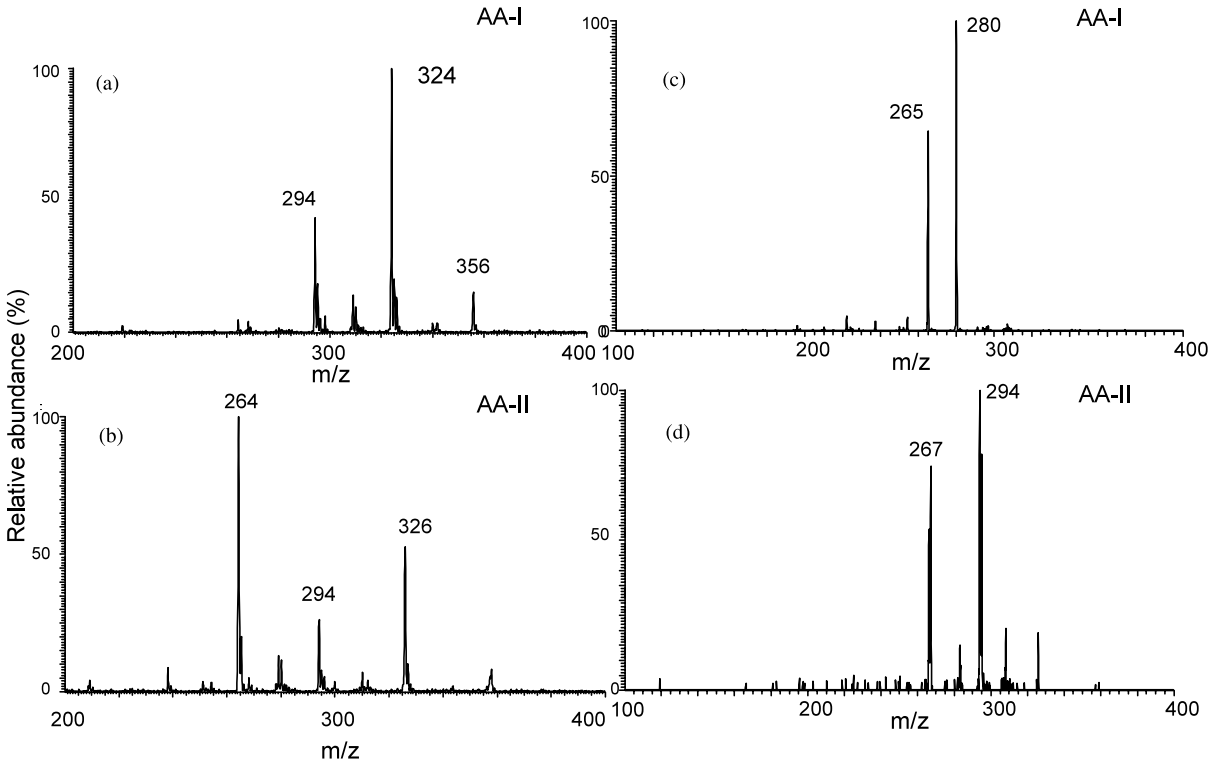

Fig. 3. Mass spectra of (a) AA-I, (b) AA-II and Product-ion spectra of (c) AA-I (precursor ion at *m/z* 324) and (d) AA-II (precursor ion at *m/z* 326), produced by LC/(+)APCI/MS/MS.

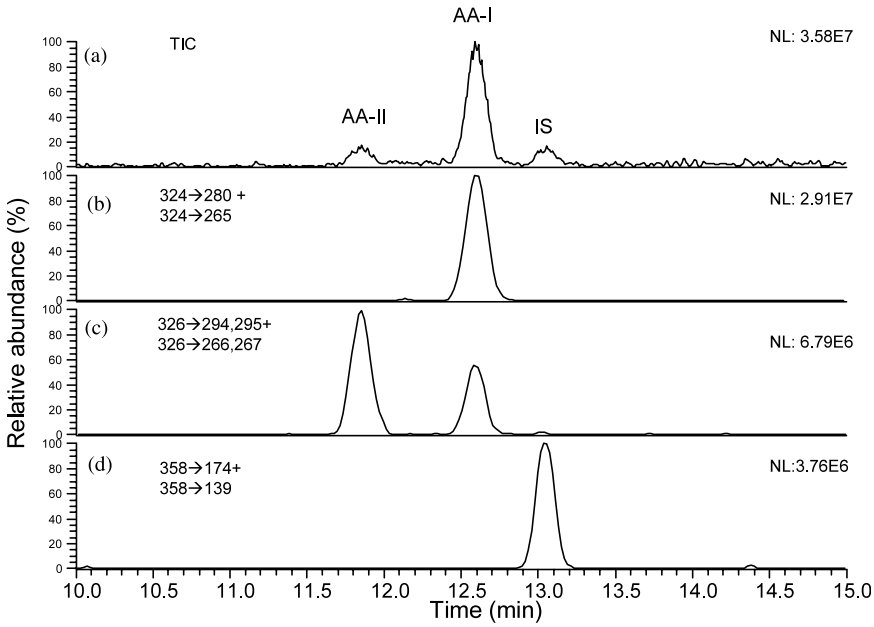

Fig. 4. Mass ion chromatograms of (a) TIC (b) AA-I (1 ppm), (c) AA-II (1.5 ppm), and (d) indomethacine (IS) (1 ppm) produced by LC/(+)APCI/MS/MS.

ion at m/z 326, and (c) indomethacine (internal standard) with m/z 358 → 174 and 358 → 139. The peaks obtained for all of the analytes had good shapes and were well separated using the LC/(+)APCI/MS/MS technique. The linearity of the peak area ratio with respect to concentration was studied at concentrations of AA-I of 10, 25, 50, 100, 250, 500, 750 and 1000 ng/ml, and the corresponding concentrations of AA-II, with indomethacine as the internal standard. Correlation coefficients of 0.9985 and 0.9971 were found for AA-I and AA-II, respectively. Although the linearity of AA-II is as good as that of AA-I, the peak area of AA-II is smaller than that of AA-I at each concentration of the standard mixtures. This observation may be because AA-II fragments into three main peaks in the precursor-ion scan, a situation that is expected to decrease the sensitivity of quantification of a selected mode of reaction monitoring in the MS/MS analysis.

The precision of the LC/(+)APCI/MS/MS method was determined by conducting five consecutive identical injections of the AA-I/II sample under the optimal conditions. The reproducibility is expressed as the relative standard deviation (R.S.D.), which varies between 6.7% at a concentration of 1000 ng/ml and 13.6% at 25 ng/ml. This precision is acceptable for analyzing trace amounts of AA. The linear-range experiment provides the necessary information to estimate the limits of detection, which are based on the lowest detectable peak that has a signal-to-noise ratio of 3. The calibration curves for the analyzed compounds are linear in the concentration range from 10 to 1000 ng/ml. The detection limit of AA-I is as low as 10 ng/ml.

The effectiveness of this method in determining the amounts of AA-I and II in actual samples was tested by analyzing nine *Asarum* species collected in China. Each sample was prepared as described before. Fig. 5 displays the SRM chromatograms of (a) the total ion count (b) AA-I, (c) AA-II and (d) the internal standard, from a methanol extract of *A. crispulatum* (sample 2 in Table 1). Analysis data from the quantification of AA-I from the MeOH extracts of nine *Asarum* species was shown on Table 1. AA-II was not detected in any of the species. Three injections of each sample were made to obtain the mean values. The results suggest that *A. sieboldii* contains the least amount of AA

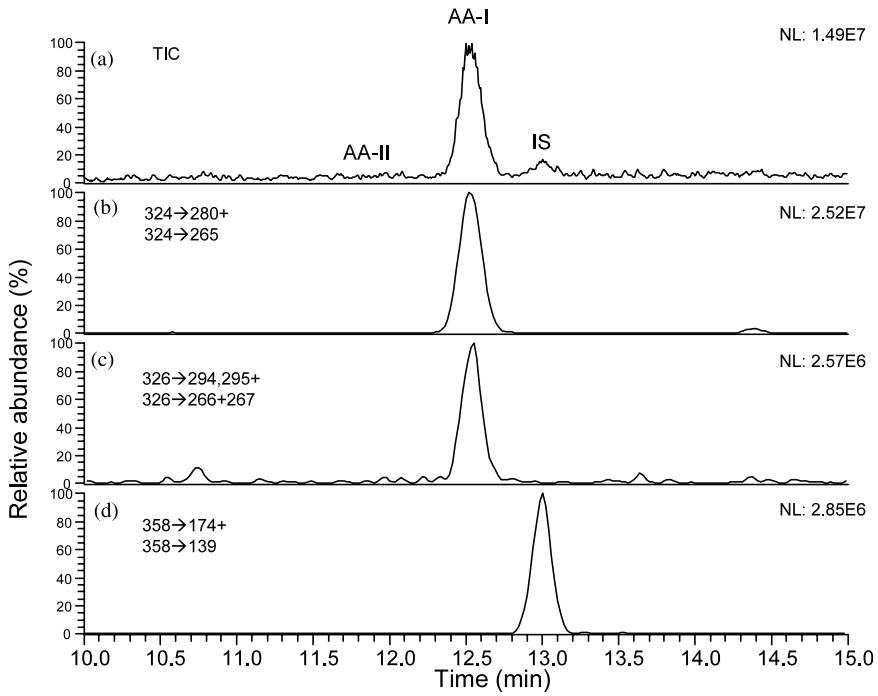

Fig. 5. SRM chromatograms of (a) TIC (b) AA-I (c) AA-II, and (d) indomethacine (IS) produced by LC/(+)APCI/MS/MS from methanol extract of *A. crispulatum*.

among the nine species and that there is a significant variation in the AA-I content between *A. sieboldii* and *A. heterotropoides* var. *mandshuricum*, which are the most common sources of the Xixin drug.

To test whether the matrix has any effect on the analyzes, the recoveries of AA-I from the extracts of three *Asarum* species (samples 1, 2 and 10 in Table 1), containing a moderate, a large and a small amount of AA-I, respectively, were determined by analyzing the extracts before and after spiking a standard of AA-I at a concentration of 250 ppb. The recoveries in these extracts were 99.5% (S.D. 5.4%), 110.2% (9.2%), and 94.1% (8.2%), respectively, which suggests that this analytical method is accurate at measuring AA-I

Table 1
AA-I concentrations (ng/mg) in nine *Asarum* spp. extracts

Entry	Sample	Sample weight (mg)	Concentration (ng/mg)	R.S.D. (%)
1	*A. heterotropoides*	16.9	42.2	7.4
2	*A. crispulatum*	3.9	3376.9	7.1
3	*A. forbesii*	5.9	105.9	12.2
4	*A. himalaicum*	2.0	17.5	11.7
5	*A. sieboldii*	9.2	3.3	12.0
6	*A. debile*	10.0	17.5	7.1
7	*A. maximum*	20.0	85.5	5.9
8	*A. ichangense*	12.5	53.4	8.4
9	*A. fukienense*	8.8	16.6	13.2
10	*A. fukienense*	74.7	11.7	7.1

Note that entries 9 and 10 are from the same species, but entry 10 represents a sample extracted with hot MeOH.

abundance in plant extracts over a wide range of concentrations.

4. Conclusions

Xixin is a very commonly used herbal medicine. LC/MS/MS was evaluated as a tool to determine the levels of trace amounts of AA-I in Xixin. Better chromatographic shapes and sensitivities were obtained by using LC/MS/MS rather than the traditional LC/UV system. By applying this technique to the determination of the amount of AA-I and II in nine *Asarum* spp. collected in China, AA-I was found to range from a low of 3.3 ng/mg in *A. sieboldii* to 3376.9 ng/mg in *A. crispulatum*.

Acknowledgements

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