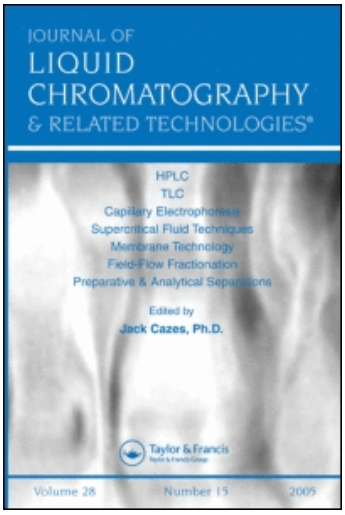

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Facile Reversed-Phase HPLC Resolution and Quantitative Determination of Aristolochic Acid and Aristolactam Analogues in Traditional Chinese Medicine

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Facile Reversed-Phase HPLC Resolution and Quantitative Determination of Aristolochic Acid and Aristolactam Analogues in Traditional Chinese Medicine

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ABSTRACT

A facile reversed-phase HPLC method for simultaneous resolution and quantitative determination of 17 analogues of aristolochic acid (AA) and aristolactam (AL) under gradient elution, with solvent mixture of sodium acetate and acetonitrile, is described. The retention scale of analytes examined in this study is found to be dependent on the pH of

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mobile phase and highly relevant to the presence of functional groups on analytes as well, under the same chromatographic conditions. Analytes with high polarity (i.e., many functional groups) elutes early. Increasing the pH of mobile phase decreases the retention time of AA analogues, however, it increases that of AL analogues slightly. At higher pH (>5.0), the elution reversal between AA and AL is observed and believed to be from the ionization of carboxyl groups on aristolochic acid. Quantitative analysis of Chinese herbs such as *Aristolochia heterophylla* and *Aristolochia fangchi* has proven this method to be facile and reliable, which can be considered as fingerprints to identify the origin or species of plant in the genus *Aristolochia.*

Key Words: Aristolochic acid; Aristolactam analogs; RP-HPLC; Traditional Chinese medicine; Resolution; Quantitative determination.

INTRODUCTION

Aristolochic acids (AAs) occur in many plants of the genus *Aristolochia*, which grow widely in the tropics to temperate areas and consist of more than 300 species. As shown in Fig. 1, these compounds are analogues of phenanthrene carboxylic acids with high similarity in structure and containing a nitro-group, a rare occurrence in nature. Some have been used as anodynes, antiphlogistics, and detoxicants in the form of herbal drugs in many cultures.[1,2] Based on the NMR study, AAs are reduced to ALs during metabolism under anaerobic conditions in vitro, and then excreted in vivo in mammals including human beings.[2] For instance, AA **6** is reduced to AL **12** during the metabolic process. Despite the potent toxic properties such as mutagenicity and a strong carcinogen in Wistar rats and mice, AAs have been used in the therapy of infectious disease, reducing blood pressure, enhancing leukocytes, and pain relief, because of their diverse biological action.[3–8]

Terminal or pre-terminal renal failure in renal disease at end stage also has been reported in a clinical study for taking weight-controlling pills with Chinese herbs, containing ingredients of AAs by mistake.[9,10] In some cases, participants developed severe nephrotoxicity and required kidney transplantation.[11] In England, two similar cases of renal failure linked to AA **6** were reported.[12,13] The cause of disease was thought to result from the preferential binding of AAs to the exocyclic amino group of purine nucleotides in DNA.[2] Note, that a mixture of AAs was still used in therapy, indicating purifying these compounds was difficult, and thus, remained to be a challenge for analytical chemists.[14] The US Food and Drug Administration (FDA) issued a warning to consumers to discontinue use of these Chinese herbal products in 2001 because they may present a serious health hazard.

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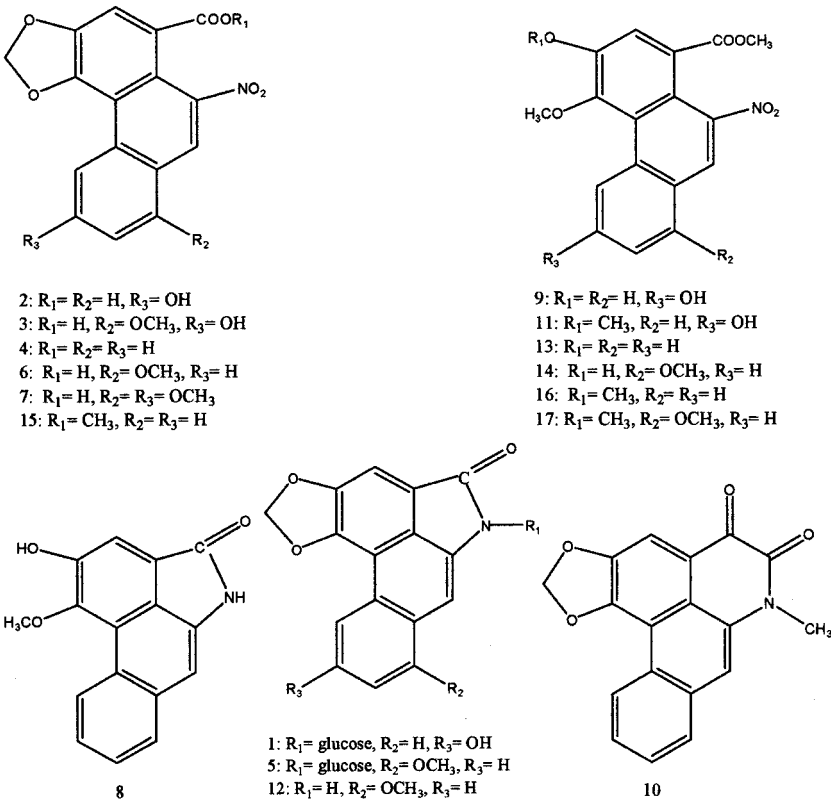

Figure 1. The structures of 17 analogues of AA and AL examined in this study.

The plant, *Aristolochia clematitis*, grows as a weed in certain area of Balkan region. It has been pointed out that the AAs from this weed may be found either in the diet or local water supplying system, through contaminated wheat with plant's seeds, or through seepage from the roots.[15] To assess the validity of this hypothesis, a facile and sensitive analysis is therefore, necessary for grain and water samples, as well as the biological samples from patients affected. The quality of botanical raw materials are found affected as well by other factors such as the soil and climate in which a plant is grown, herbivores, plant pathogens, time of harvest, and drying and/or storage conditions.[16–18]

The quantitative determination of AAs was first conducted through the photometry or spectrometry approach.[19,20] Rao et al. resolved methylated AAs via the GLC approach, and then performed the fluorometric assay based on the reduction to lactams.[15] Mamolo and others reported the HPLC resolution of

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AAs in the reversed-phase mode.[14,21–25] A group of seven ALs was first separated by Makuch et al. in 1992, under gradient elution with solvent mixture of phosphoric acid buffer and acetonitrile.[26] However, to the best of our knowledge, the simultaneous resolution of analogues of AA and AL with this magnitude of complexity, including five AAs (**2**–**4**, **6**, and **7**), two AL glucosides (**1** and **5**), two ALs (**8** and **12**), six ariskanines (**9**, **11**, **13**, **14**, **16**, and **17**), and one cepharadione, has not yet been reported. In this study, we demonstrate the reversed-phase HPLC resolution of seventeen analogues of AA and AL under gradient elution and prove this method to be facile, reliable in routine analysis of Chinese herbs, and with a possibility of being used as fingerprints to identify the origin or species of plant in the genus *Aristolochia.*

EXPERIMENTAL

Apparatus

The HPLC system that was used to carry out all analyses was a Gilson model 305 linked to a Gilson Model 115 UV detector with variable wavelengths. The UV detection was set at 254 nm. All calculations based on the peak area were processed on a Hitachi D-2000 data integrating station. A 20 μL injection valve (Rheodyne) was used in all analyses. The separation was carried out on a C_{18} column (250 × 4.6 mm i.d., 5 μm particle diameter, supplied by E. Merck) at ambient temperature (~28°). A C_{18} column (250 × 10.0 mm, i.d.) was used to perform semi-preparative purification.

Chemicals

Solvents of HPLC grade such as methanol and acetonitrile were from BDH (England). Dichloromethane, glacial acetic acid, and sodium hydroxide were purchased from E. Merck (Germany). In all cases, water processed with a Millipore water purifying system was used.

Preparation of Standards and Sample Solutions

Plant extracts of *Aristolochia cucurbitafolia* and *Aristolochia kankauensis* were the source for all standards examined in this study.[27,28] The crude extract was first TLC separated and then further purified with a semi-preparative column (250 × 10.0 mm, i.d.). Eluents were collected, concentrated, and then dried under reduced pressure in a rotary evaporator. The dried components were not purified further and readily dissolved in a solvent mixture of dichloro-

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methane and methanol (1 : 1, v/v) with concentrations as follows: 10.0 μg/mL (compounds **1** and **11**), 5.0 μg/mL (compounds **2–7**, **10**, **12**, and **16**), 4.0 μg/mL (compound **13**) and 2.5 μg/mL (compounds **8**, **9**, **14**, **15**, and **17**).

Roots and stems of *Aristolochia heterophylla* and *Aristolochia fangchi* were weighed 1.0 g, respectively and cut into small pieces before being refluxed in methanol of HPLC grade. The methanol extract was collected, filtered, and then diluted to 5 mL and was used as sample solution for HPLC analysis.

Chromatographic Conditions and Calibration

The separation of AAs and ALs was carried out on a 25 cm C_{18} column using gradient elution with a solvent mixture of sodium acetate (mobile

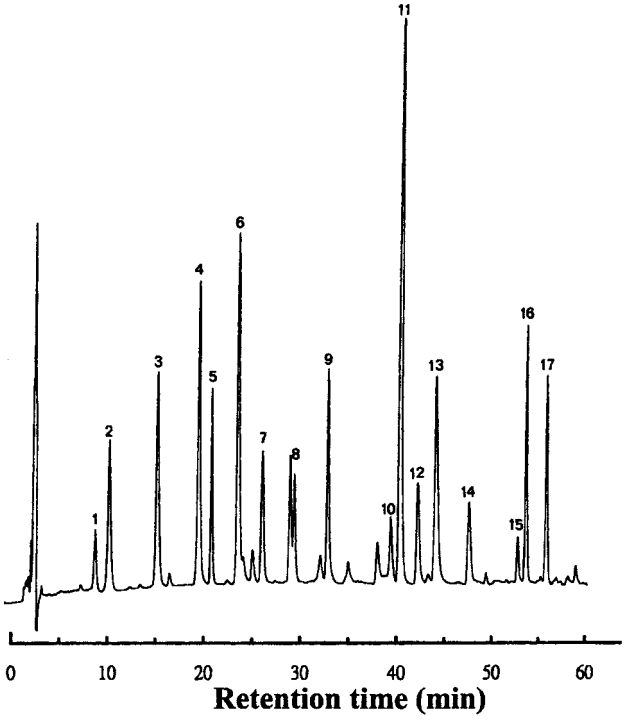

Figure 2. Chromatogram showing the resolution of seventeen AA and AL analogues under gradient elution with a solvent mixture of sodium acetate and acetonitrile. Compound corresponding to the number designated in the elution order is described in Fig. 1. Please refer to Experimental Section for the detail of gradient elution. Peaks not assigned are impurities, which fail to be removed by the semi-preparative column.

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phase A: 0.01 M, pH 5.0) and acetonitrile (mobile phase B) at ambient temperature (~28°C). The gradient elution started with 20% B for 5 min. The percentage of mobile phase B was increased to 44% at 33 min, 50% at 43 min, then to 68% at 53 min, and finally to 80% at 59 min.

Each standard was prepared in three different concentrations (14–205 μg/mL), which were plotted against corresponding peaks area to create calibration curves. The measurements were all in triplicate. Linear regression analysis was performed to calculate the value of square *R*. The method of external standard addition was applied to quantitatively determine the concentration of AA and AL analogues in *A. heterophylla* and *A. fangchi.*

RESULTS AND DISCUSSION

The resolution of seventeen AA and AL analogues under gradient elution with a solvent mixture of sodium acetate and acetonitrile is shown in Fig. 2. Peaks not assigned are impurities, which fail to be removed by a semi-preparative column. The value of square *R* for standards examined in this study is listed in Table 1. As can be seen, concentration of standards is well

Table 1. R^2 values for 17 standards of AAs and AL.

Compound no.	Name	$R^{2\,a}$
1	Aristolactam-C-*N*-*β*-glucoside	0.9992
2	Aristolochic acid C	0.9982
3	Aristolochic acid IVa	0.9987
4	Aristolochic acid II	0.9994
5	Aristolactam-*N*-*β*-D-glucoside	0.9999
6	Aristolochic acid I	0.9990
7	Aristolochic acid IV	0.9999
8	Aristolactam AII	0.9999
9	Ariskanin B	0.9999
10	Cepharadione A	0.9993
11	Ariskanin C	0.9999
12	Aristolactam I	0.9998
13	Ariskanin ?	0.9998
14	Ariskanin D	0.9999
15	Aristolochic acid II methyl ether	0.9990
16	Ariskanin A	0.9982
17	Ariskanin E	1.0000

[a]Standard was prepared in three different concentrations and measured in triplicate at each concentration.

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correlated with the peak area within the calibration range in the linear regression.

The retention scale of analytes is found dependent on the pH of mobile phase and highly relevant to the analyte's polarity (i.e., the presence of functional groups) as well, under the same chromatographic conditions. Figure 3 shows increasing the pH of mobile phase decreases the retention time of AAs, however, it increases that of ALs slightly. At higher pH (>5.0), the elution reversal between AA and AL is observed and believed to be from the ionization of the carboxyl group on AA. Aristolactam analogues do not contain carboxyl group and, thus, are less sensitive to the change in pH. Also, analytes with high polarity (i.e., AA and analogue with a hydroxyl group attached) appears to elute early. As expected, ALs and ariskanins are eluted late. In light of this, the optimal resolution for AA and AL analogues can be obtained using 0.01 M sodium acetate solution buffered at pH 5.0 as the mobile phase upon extrapolation.

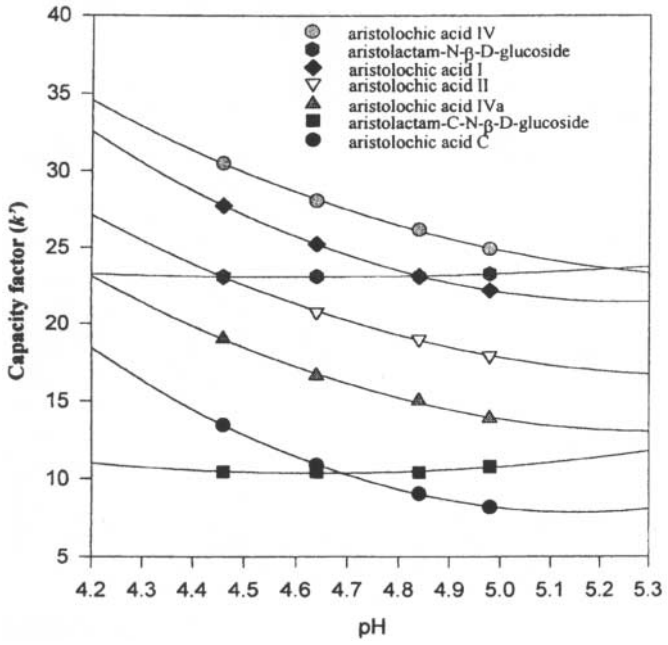

Figure 3. The effect of pH of mobile phase on the retention scale of analyte under gradient elution as described in the Experimental Section. As can be seen, increasing the pH of mobile phase decreases the retention time of AA analogues, however, increases that of AL analogues slightly. At higher pH (>5.0), the elution reversal between AA and AL is observed and believed to result from the ionization of carboxyl group on AA.

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Chromatograms for the resolution of the first seven eluted AA and AL analogues in methanol crude extracts of *A. heterophylla* and *A. fangchi* under the same chromatographic conditions, are shown in Figs. 4 and 5. The data for quantitative analysis are summarized in Table 2. As can be seen, the profile of chromatograms, as well as the compositions and, thus, corresponding concentration of AA and AL analogues present in the methanol extracts are quite different. Note, that analyte **5** found in *A. heterophylla* is not present in *A. fangchi.* However, in the case of analyte **15**, the opposite is observed. This unique feature can be applied to determine the origin or the species of plant in the genus *Aristolochia.*

CONCLUSION

Resolution and quantitative determination of 17 analogues of AAs and ALs under gradient elution, with a solvent mixture of sodium acetate and

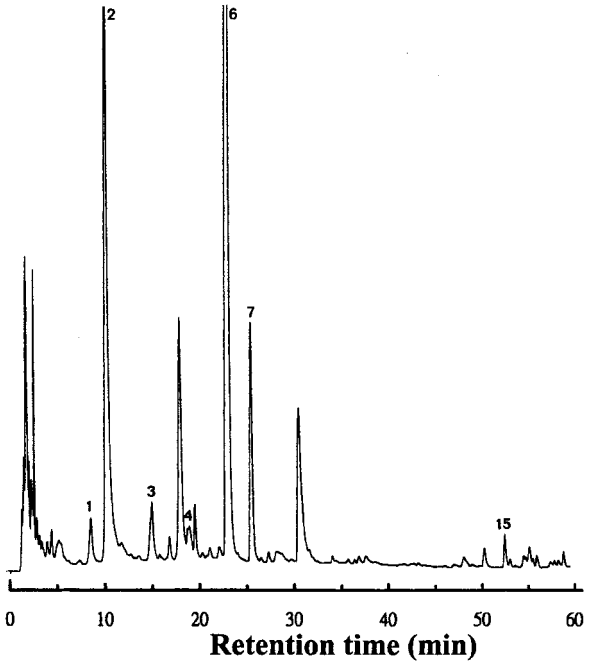

Figure 4. Chromatogram showing the resolution and quantitative analysis of seven AA analogues in the methanol extract of *A. heterophylla* under the same chromatographic conditions as described in Fig. 2.

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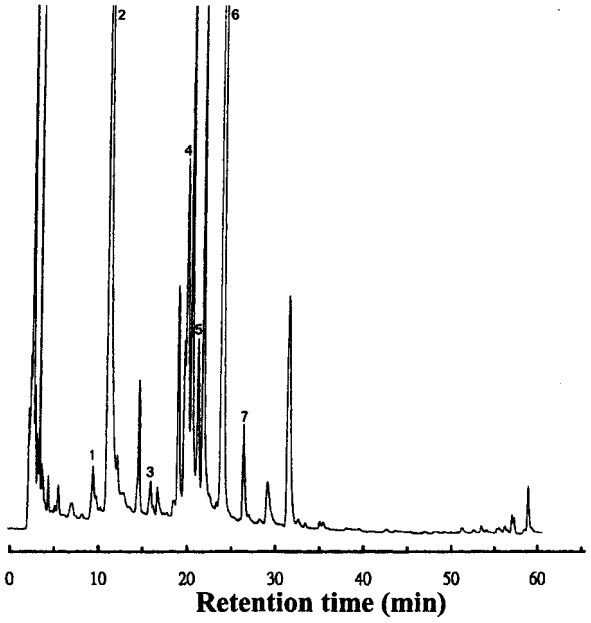

Figure 5. Chromatogram showing the resolution and quantitative analysis of seven AA analogues in the methanol extract of *A. fangchi* under the same chromatographic conditions as described in Fig. 2. As can be seen, the profile of chromatogram is quite different from that for *A. heterophylla* in Fig. 4.

acetonitrile, is demonstrated. The retention scale is found to be dependent on the pH of mobile phase and highly relevant to the analyte's polarity, as well, under the same chromatographic conditions. Analytes with high polarity appear to elute early. Increasing the pH of mobile phase decreases the retention time of AA analogues, however, it increases that of AL analogues slightly. At higher pH (>5.0), the elution reversal between AA and AL is observed and believed to result from the ionization of a carboxyl group on

Table 2. Composition and concentration of AAs and ALs.

AA and AL	**1** (%)	**2** (%)	**3** (%)	**4** (%)	**5** (%)	**6** (%)	**7** (%)	**15** (%)
A. heterophylla	14.3	43.1	3.5	0.5	N[a]	20.8	14.7	3.1
A. fangchi	16.2	44.8	1.9	7.7	8.1	14.5	6.9	N[a]

[a]Not detected.

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AA. High correlation between concentration and peak area in quantitative analysis of Chinese herbs such as *A. heterophylla* and *A. fangchi* has proven this method to be facile and reliable, which provides fingerprints to identify the origin or species of plant in the genus *Aristolochia.*

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