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Analysis of aristolochic acids, aristololactams and their analogues using liquid chromatography tandem mass spectrometry

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[ABSTRACT] More than 80 aristolochic acids (AAs) and aristololactams (ALs) have been found in plants of the Aristolochiaceae family, but relatively few have been fully studied. The present study aimed at developing and validating a liquid chromatography tandem mass spectrometry (LC/MS^n) for the analysis of these compounds. We characterized the fragmentation behaviors of 31 AAs, ALs, and their analogues via high performance liquid chromatography coupled with electrospray ionization mass spectrometry. We summarized their fragmentation rules and used these rules to identify the constituents contained in *Aristolochia contorta*, *Ar. debilis*, *Ar. manshurensis*, *Ar. fangchi, Ar. cinnabarina,* and *Ar. mollissima.* The AAs and ALs showed very different MS behaviors. In MS^1 of AAs, the characteristic pseudomolecular ions were $[M + NH_4]^+$, $[M + H]^+$, and $[M + H - H_2O]^+$. However, only $[M + H]^+$ was found in the MS^1 of ALs, which was simpler than that of AAs. Distinct MS^n fragmentation patterns were found for AAs and ALs, showing the same skeleton among the different substituent groups. The distribution of the 31 constituents in the 6 species of *Aristolochia* genus was reported for the first time. 25 Analogues of AAs and ALs were detected in this genus. A hierarchical schemes and a calculating formula of the molecular formula of these nitrophenanthrene carboxylic acids and their lactams were proposed. In conclusion, this method could be applied to identification of similar unknown constituents in other plants.

[KEY WORDS] LC/MS^n; Aristolochic acids; Aristololactams; Fragmentation rules; *Aristolochia* genus

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Introduction

Aristolochic acids (AAs) and aristololactams (ALs) are broadly distributed, but not confined, in plants of the Aristolochiaceae family. The AAs are contained mostly in the Aristolochiaceae and sometimes isolated from other plants [1-4] and some types of butterflies fed on Aristolochiaceae plants [5-6]. ALs can also be isolated from other plants besides Aristolochiaceae, such as Annonaceae, Monimiaceae, Menispermaceae, and Piperaceae.

Some aristolochic acids had been associated with the development of progressive nephropathy and other kidney damages in humans [7-9] and some aristololactams were known to be cytotoxic to human proximal tubular epithelial cells (HK-2) [10-11]. Recent studies [12-14] have reported the aristolochic acid-induced nephrotoxicity and demonstrated the underlying mechanism of action. Therefore, the evaluation of medicinal safety of crude drugs containing AAs and ALs in this family is very important for the protection of public health.

In recent years, attempts have been made to establish an analysis method for AAs and ALs, concerning the renal toxicity of some of these constituents. The reported methods

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Abbreviations AAs: aristolochic acids, ALs: aristololactams, TLC: thin layer chromatography, UV: ultraviolet spectrometry, CE: capillary electrophoresis, ELISA: enzyme-linked immunosorbent assay, HPLC-DAD: high-performance liquid chromatography coupled to photo diode array detector, HPLC-FLD: high-performance liquid chromatography coupled to fluorescence detector, HPLC-UV-MS: high-performance liquid chromatography coupled to both UV and mass spectrometry, 7-OCH_3-AL-IV: 7-methoxy-aristololactam IV, AL-IVa: aristololactam IVa, AA-I: aristolochic acid I, ESI: electrospray ionization, APCI: atmospheric pressure chemical ionization, TFA: trifluoroacetic acid, THF: tetrahydrofuran
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include thin layer chromatography (TLC) [15-16], ultraviolet spectrometry (UV) [17], capillary electrophoresis (CE) [18-19], enzyme-linked immunosorbent assay (ELISA) [20], high-performance liquid chromatography coupled to photo diode array detector (HPLC-DAD) [21-23], fluorescence detector (HPLC-FLD) [24] and both UV and mass spectrometry (HPLC-UV-MS) [25-27]. For the sensitive and specific detection as a powerful approach to identification and quantification of constituents in medicinal plants and their preparations, the HPLC-DAD-MS method shows certain advantages compared with other methods.

However, most papers have reported on only the analysis of a few AAs and/or ALs. Since more than 80 AAs and ALs are widely distributed in this Aristolochiaceae family [1], more detailed studies of these AAs, ALs and their analogues are thus urgently needed. These clues prompted us to develop an HPLC-DAD-MS method for the simultaneous identification and analysis of derivatives of AAs and ALs.

In our previous research [11], we find that cytotoxicity of aristololactam IVa (AL-IVa) and 7-methoxy-aristololactam IV (7-OCH_3-AL-IV) extracted from *Aristolochia. contorta* are similar to or even stronger than that of aristolochic acid I (AA-I), which has cytotoxic activity in renal HK-2 cells in both MTT and LDH leakage assays (5 μg·mL^{-1}). In this paper, we reported a sensitive and specific method for the analysis of related compounds in this nitrophenanthrene category. The MS behaviors of 31 AAs, ALs and their analogues were investigated exhaustively, and their MS^1 to MS^5 fragmentation rules were deduced. These rules were then used to identify the related constituents contained in 6 *Aristolochia* plants of the Aristolochiaceae family.

Materials and Methods

Chemicals and materials

Extracts of *Aristolochia fangchi* Y. C. Wu ex L. D. Chow et S. M. Hwang, *Aristolochia contorta* Bunge, *Asarum maximum* Hemsl, *Saruma henryi* Oliv. and *Thottea hainanensis* (Merr. et Chun.) D. Hou, extracted by the authors, were the primary sources for all chemical standards. Some chemical standards were synthesized by the authors from aristolochic acids I and II according to literatures [28-31]. Phenanthrene was purchased from Sigma-Aldrich (St. Louis., MO, USA) (Lot: A0234070). Their detailed information (Table 1) and structures are shown below.

Table 1 Nomenclature, molecular weights, sources and retention times of chemical standards utilized in this research

No.	Chemical Name	Abb. Name	M_w	Source	t_R
1	aristololactam IIIa-*N*-*β*-D-glucoside	AL-IIIa-Glc	441	herb of *Thottea hainanensis*	12.5
2	aristolochic acid IIIa-*O*-*β*-D-glucoside	AA-IIIa-Glc	489	root of *Aristolochia contorta*	14.7
3	aristololactam Ia-*N*-*β*-D-glucoside	AL-Ia-Glc	441	root and rhizome of *Asarum heterotropoides* var. *mandshuricum*	18.2
4	aristolochic acid IVa-*O*-*β*-D-glucoside	AA-IVa-Glc	519	root of *Ar. contorta*	18.7
5	norcepharadione A-*N*-*β*-D-glucoside	norcep-A-Glc	453	root and rhizome of *As. heterotropoides* var. *mandshuricum*	18.7
6	aristololactam II- *N*-*β*-D-glucoside	AL-II-Glc	425	root of *Ar. contorta*	22.9
7	aristololactam I-*N*-*β*-D-glucoside	AL-I-Glc	455	root of *Ar. contorta*	25.7
8	aristololactam IIIa	AL-IIIa	279	synthesis from aristolochic aicd IIIa	29.8
9	aristololactam Ia	AL-Ia	279	synthesis from aristololactam I	32.9
10	aristolochic acid IIIa	AA-IIIa	327	fruit of *Ar. contorta*	33.4
11	aristololactam AII	AL-AII	265	herb of *Saruma henryi* Oliv.	33.7
12	aristololactam FI	AL-FI	265	stem and root of *T. hainanensis*	35.4
13	aristolochic acid VIIa	AA-VIIa	357	fruit of *Ar. contorta*	35.8
14	aristolochic acid IVa	AA-IVa	357	stem and leaf of *Ar. contorta*	36.2
15	7-methoxy-aristololactam IV	7-OCH_3-AL-IV	353	herb of *As. caulescens*	42.4
16	cepharadione A	cepharadione-A	305	stem and root of *T. hainanensis*	42.8
17	aristololactam VII	AL-VII	323	root and rhizome of *As. maximum*	42.9
18	aristololactam II	AL-II	263	stem and leaf of *Ar. contorta*	43.5
19	aristolochic acid II	AA-II	311	root of *Ar. fangchi*	48.9
20	aristololactam I	AL-I	293	stem and leaf of *Ar. contorta*	49.5
21	9-ethoxy-aristololactam I	9-OC_2H_5-AL-I	337	root and rhizome of *As. heterotropoides* var. *mandshuricum*	50.1
22	9-ethoxy-aristololactam IV	9-OC_2H_5-AL-IV	367	root and rhizome of *As. heterotropoides* var. *mandshuricum*	50.2