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A novel and specific method for the determination of aristolochic acid-derived DNA adducts in exfoliated urothelial cells by using ultra performance liquid chromatography–triple quadrupole mass spectrometry

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ABSTRACT

Aristolochic acid nephropathy (AAN) is associated with the prolonged exposure to nephrotoxic and carcinogenic aristolochic acids (AAs). DNA adducts induced by AAs have been proven to be critical biomarkers for AAN. Therefore, accurate and specific quantification of AA–DNA adducts is important. In this study, a specific method using ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed and applied for the determination of 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) in exfoliated urothelial cells of AA-dosed rats. After the isolation from urine samples, DNA in urothelial cells were subjected to enzymatic digestion and solid-phase extraction on a C_{18} Sep-Pak cartridge for the enrichment of DNA adducts. The sample extracts were analyzed by reverse-phase UPLC–MS/MS with electrospray ionization in positive ion mode. The quantification of the AA–DNA adduct was performed by using multiple reaction monitoring with reserpine as internal standard. The method provided good accuracy and precision with a detection limit of 1 ng/ml, which allowed the detection of trace of dA-AAI in exfoliated urothelial cells. After one-month oral dose of AAI at 10 mg/kg/day, 2.1 ± 0.3 dA-AAI per 10^9 normal dA was detected in exfoliated urothelial cells of rats. Compared to the traditional methods such as ^{32}P-postlabelling and HPLC with fluorescence detection, the developed UPLC–MS/MS method is more specific and rapid with a retention time of 4 min. The outcome of this study may have clinical significance for diagnosing and monitoring AA-associated disease because detection of DNA adducts in exfoliated urothelial cells is non-invasive and convenient.

1. Introduction

Aristolochic acid nephropathy (AAN), a unique type of rapid progressive renal fibrosis frequently associated with urothelial malignancy, was reported in a group of patients in Belgium who had ingested slimming pills containing *Aristochia fangchi*. Half of the patients needed renal replacement therapy, including renal transplantation [1–5]. 8-Methoxy-6-nitro-phenanthro-[3,4-d]-1,3-dioxole-5-carboxylic acid (Aristolochic acid I, AAI) and its 8-demethoxylated derivative (Aristolochic acid II, AAII) (Fig. 1) existing in many herbal plants have been proven to be the source of AAN [6–8]. The biotransformation of AA has been investigated by analyzing the tissues of patients with AAN [9–11] and AA-dosed rodent cells [12]. A metabolic pathway involving the reduction of nitro group was suggested [13]. As shown in Fig. 1, AAI and AAII were metabolized to a cyclic aristolactam nitrenium ion by the activation of several mammalian enzymes. The resulted intermediate with a delocalized positive charge was electrophilic, which might bind preferentially to the exocyclic amino groups of purine or cytosine nucleotides in DNA through the C-7 position of the phenanthrene ring, leading to the formation of AA–DNA adducts. The major AA–DNA adducts found in rodents exposed to AA and in patients suffering from AAN were identified as 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AAI), 7-(deoxyguanosin-N^6-yl) aristolactam I (dG-AAI), 7-(deoxyadenosin-N^6-yl) aristolactam II (dA-AAII), and 7-(deoxyguanosin-N^6-yl) aristolactam II (dG-AAII). The DNA adducts have been used as biomarkers for the exposure to AA to investigate the mutagenic and carcinogenic potentials of AA. Therefore, detection and quantification of AA–DNA adducts would have significant implications for the disease risk assessment.

However, the analysis of AA–DNA adduct at low levels in a complex biological matrix including protein, ribonucleic acid, and salt as well as the excess unmodified bases, has been the major analytical challenge. A method suitable for the analysis of AA–DNA adducts should simultaneously tolerate the constraint of limited sample availability and the need on lower detec-

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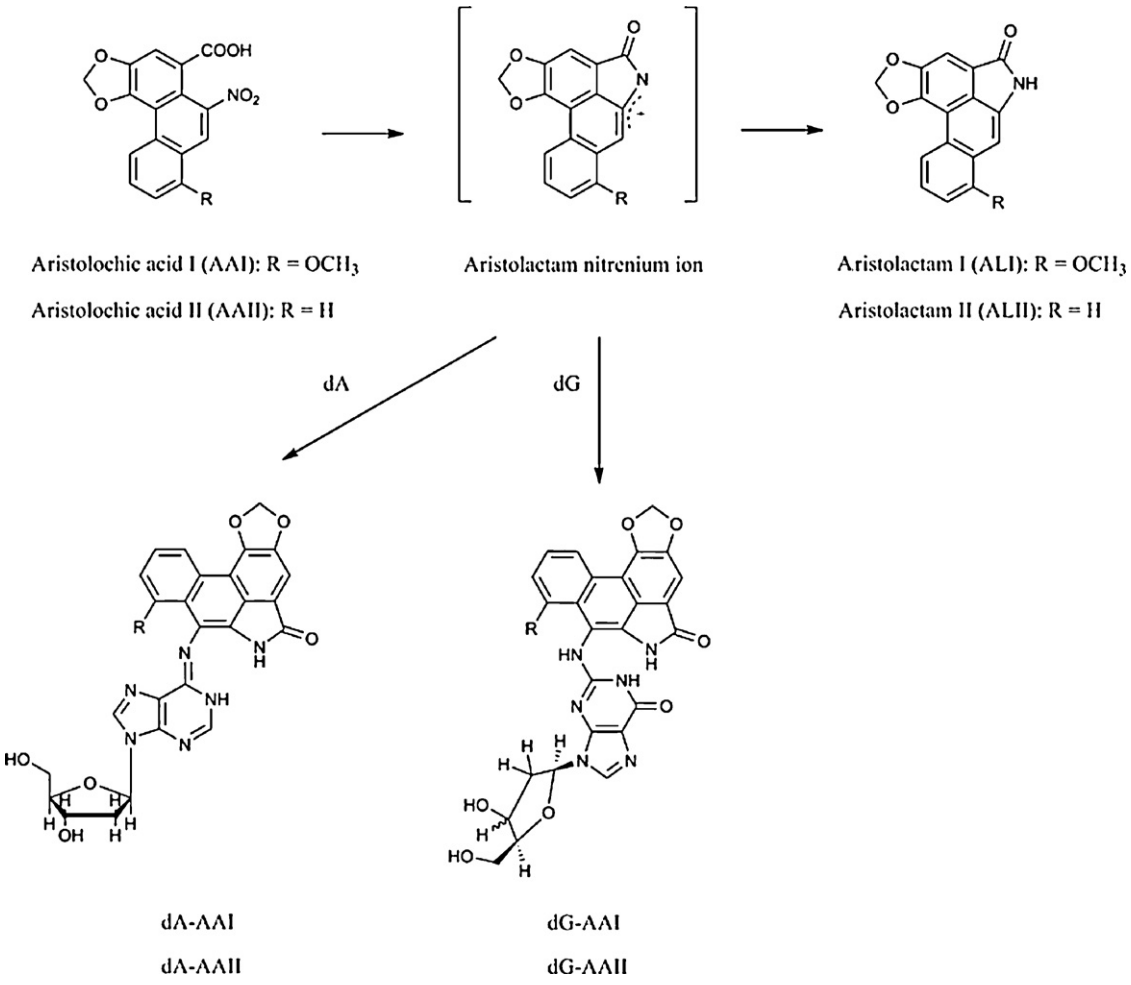

Fig. 1. Metabolic activation and DNA adduct formation of aristolochic acid I (AAI, R = OCH_3) and II (AAII, R = H).

tion limits. A number of methods have been developed for the detection of AA–DNA adducts, including ^{32}P-postlabelling analysis [14–17], high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) [18–20] and fluorescence detection (HPLC-FLD) [21]. The ^{32}P-postlabelling assay is an ultrasensitive method and capable of detecting adducts at levels as low as 1 in 10^9 nucleotides. However, it required the use of large excess of radioactive γ-^{32}P labeled orthophosphate that limited its application only in special laboratories with the control of radioactive material and that is a strong β-emitter. While HPLC-FLD allowed a sensitive detection of dA-AAII with a low detection limit of 18.3 fmol on column by monitoring the fluorescence intensity of dA-AAII [21], all of AA–DNA adducts contained the same fluorophore aristolactam and thus exhibited the similar fluorescence excitation and emission spectra, which made the method less specific.

Compared to conventional HPLC analysis, ultra performance liquid chromatography (UPLC) offered the improved chromatographic resolution and increased peak capacity through a rapid elution on a column packed with 1.7 μm particles. The higher efficiency of small particles enabled shorter columns to be used, reducing analysis time and solvent consumption. In mass spectrometry analysis, the use of multiple-reaction-monitoring (MRM) mode selected and monitored both parent and one or more product ions simultaneously and thus provided better specificity and sensitivity over the selected-ion-monitoring and full-scan detection modes. In the present work, we aimed at developing a specific and rapid method for the detection of low level AA–DNA adduct in exfoliated urothelial cells by exploring the possibilities of combining the advantages of UPLC and MRM. To the best of our knowledge, this work presented the first application of UPLC in the analysis of AA–DNA adducts. The detection of AA–DNA adduct in exfoliated urothelial cells may be of clinical significance for diagnosing and monitoring on AA-associated poisoning and diseases because the analysis is non-invasive and convenient. Schmeiser et al. suggested that dA-AAI, the predominant AA–DNA adduct *in vivo*, might be the critical mutation in the carcinogenic process in rodents [22,23]. Therefore, this study focused the method development for dA-AAI as biomarker for monitoring the AA exposure.

2. Experimental

2.1. Chemicals and reagents

Aristolochic acid containing 97% AAI was purchased from Acros (NJ, USA). Reserpine, 2-deoxyadenosine (dA), DNase I, phosphodiesterase I and alkaline phosphatase were purchased from Sigma (St. Louis, MO, USA). Ammonium hydroxide solution was obtained from Acros (NJ, USA). Diethyl ether (DEE), methanol (MeOH) and HPLC-grade acetonitrile were purchased from Tedia (Fairfield, OH, USA). Milli-Q water (18.2 MΩ) was prepared using a Milli-Q Ultrapure water purification system (Millipore, Billerica, USA).

2.2. Synthesis of dA-AAI adduct

Following a modification of the protocol of Schmeiser et al. [24,25], 20 μmol of AAI was first converted to corresponding