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Rapid and specific determination of DNA adducts for clinical diagnosis of poisoning and disease associated with aristolochic acid

Key Messages

1. A liquid chromatography–mass spectrometry method was developed to detect and characterise aristolochic acid–DNA adducts in biological samples.
2. The detection of DNA adducts in plasma, urine or the cells found in urine may be useful to support the diagnosis and monitoring of aristolochic acid–associated poisoning and disease.
3. Efforts should be made to improve the sensitivity and specificity of this approach for the detection and characterisation of exposure to other mutagens/carcinogens.

Introduction

Exposure to aristolochic acids (AAs), derived from the herbal genus *Aristolochia* and *Asarum*, is associated with the development of nephropathy (aristolochic acid nephropathy). Prolonged exposure to AAs has been reported from medicinal applications and the use of AA-related herbs in slimming products. Upon enzyme activation, AAs are metabolised to the aristolactam-nitrenium ion intermediates leading to the formation of the corresponding DNA adducts. Therefore, such DNA adducts may serve as biomarkers of exposure and can be used to confirm ingestion of AAs. Analysis of DNA-AA adducts may support the clinical diagnosis of AA-associated poisoning and disease.

Although the import and sale of herbs *Aristolochia* and related products containing AAs have been banned in Hong Kong since 1 June 2004, certain AA-containing herbs that yield small amounts are still available. Moreover, there is confusion concerning which herbs contain AAs. It is unknown how many people have been exposed to these potentially dangerous herbs and for how long. The possible consequences of long-term exposure to AAs include renal failure and urinary tract cancers. It is therefore important to monitor the long-term outcome of exposure to AAs.

Damage to DNA due to prolonged exposure to AAs significantly increases the risk of cancer in humans. Diagnosis of acute poisoning from AAs is possible, but a retrospective diagnosis is difficult to make. With respect to research on AA-induced kidney disease, a simple and rapid method for detecting DNA-AA adducts is needed. This study aimed to develop a novel assay for analysis of such DNA adducts of AAs in biological samples. The liquid chromatography–mass spectrometry (LC-MS) method was validated and applied to analyse DNA adducts in human kidney tissues, in addition to plasma and urine samples collected from rats dosed with AAs. The feasibility of applying the developed method to study adduct formation in white cell DNA was investigated, and the potential clinical use of such adducts as biomarkers in blood or urine, instead of kidney tissue, was explored.

Methods

This study was conducted from January 2008 to December 2009. In vitro experiments were conducted to produce various DNA adducts of AAs. Sample matrices ranging from oligonucleotides, calf thymus-DNA to animal DNA were used. Animal studies involving oral administration of AAs to rats were conducted to study of AA toxicity. The induction of AAs in single and multiple dosing experiments were carried out. Determination and characterisation of known and unknown DNA adducts of AAs was conducted by exposing rats to AAs via the oral and intravenous route. The formation of AA-DNA adducts in liver and kidney tissue, as well as biofluids (eg urine and plasma) was evaluated. Rat kidney tissues were collected, with a view to extracting and isolating DNA according to the animal study protocols. Liver and kidney tissue samples were collected for the analysis of DNA-AA adducts using the developed LC-MS method. After

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method validation, the LC-MS method was also applied for the analysis of rat plasma and urine samples.

Procedures of sample pre-treatment and LC-MS analysis of DNA-AA adducts were developed. Direct analysis of urine samples and simple protein precipitation for plasma samples were combined with solid-phase

extraction enrichment and cleanup procedures. Protocols were established to pool certain urine samples together in order to achieve sufficient quantities for analysis. The biological sample extracts were analysed using the LC-MS methods using an existing LC/ion trap mass spectrometer and an LC/quadrupole time-of-flight mass spectrometer. LC separation was important for achieving adequate sensitivity and specificity. For mass spectrometry analysis, both negative and positive electrospray ionisations, along with various instrumental parameters, were investigated and optimised. The obtained LC-MS data on DNA-adducts were validated using traditional ^{32}P -post-labelling analysis.

Results

The *in vitro* experiments with oligonucleotides and AAs were successful in producing DNA adducts. The DNA adducts produced via *in vitro* experiments were characterised by electrospray ionisation tandem mass spectrometry. Thus, LC-MS/MS was found to be a powerful technique for the identification and positional mapping of the DNA adducts in oligonucleotides.¹ The developed method was successfully applied to the analysis of unmodified and AA-modified oligonucleotides (Fig 1). The observation of the modified bases (modified adenine and guanine) together

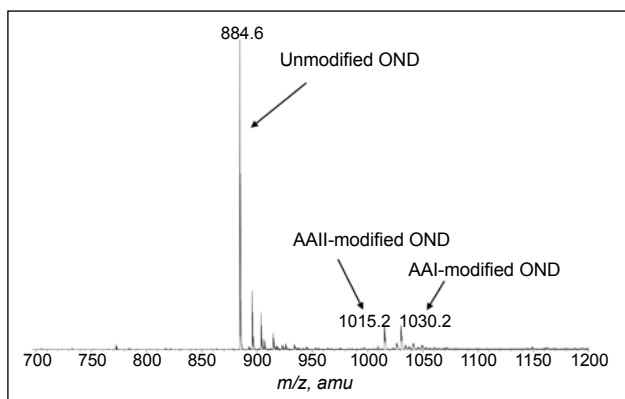


Fig 1. Typical ESI-MS spectrum of 5'-TTTATT-3' modified with 35 equivalents of aristolochic acids (AA) [mixture of AAI and AAIL]. Labelled peaks display m/z values of the $[\text{M}-2\text{H}]^{-}$ ions of the unmodified and AA-modified ODNs.

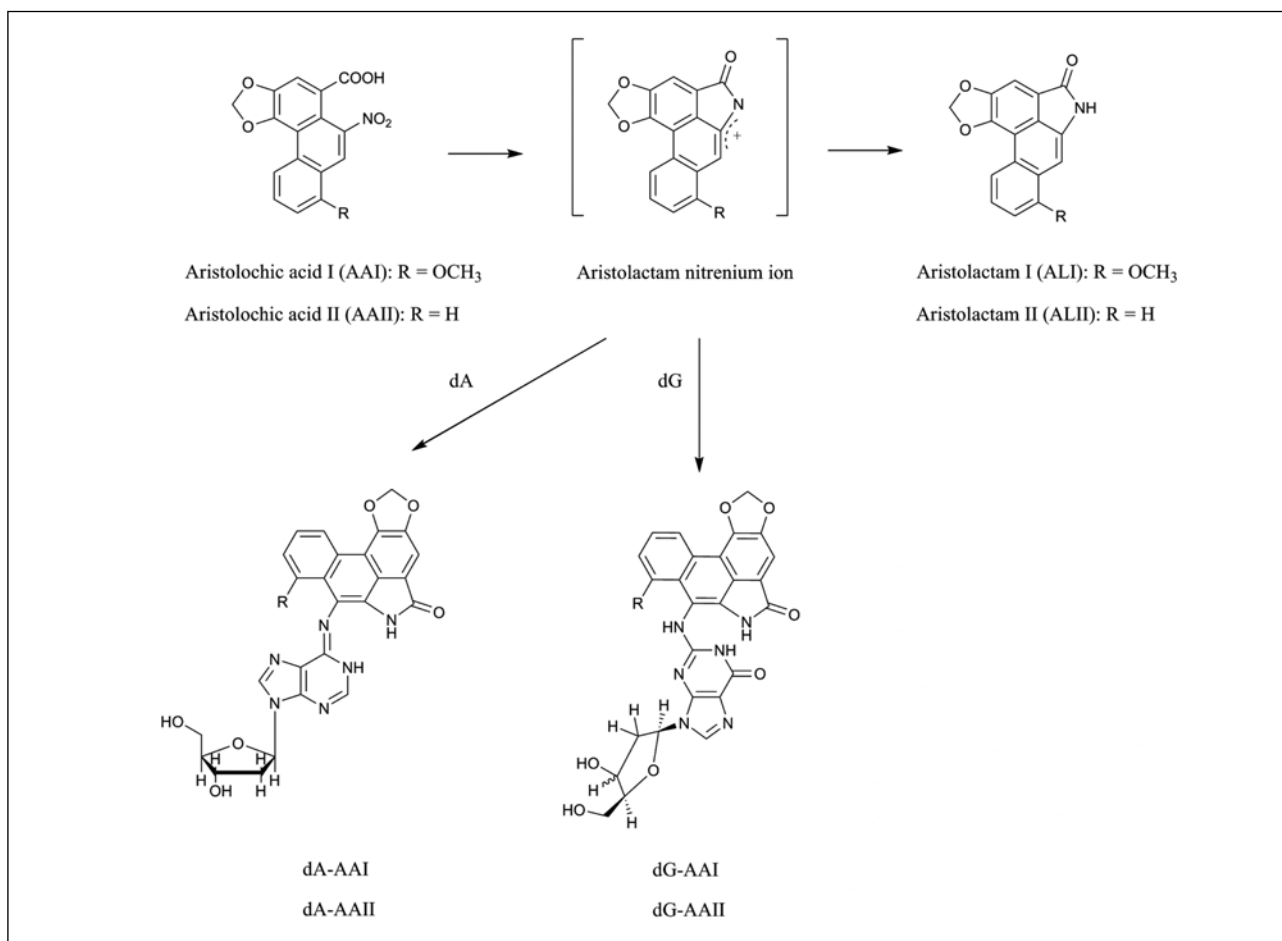


Fig 2. Metabolic activation and DNA adduct formation of aristolochic acids.

with the complementary product ions ($[a_n - B_n^+]$, w-) from the cleavage of the 3' C-O bond adjacent to the modified base in MS/MS analyses readily enabled the identification of the AA binding site in oligonucleotides.

The metabolic activation of AAs was investigated. In vitro metabolism studies indicated that AAs were metabolised to *N*-hydroxyaristolactam, which could be either reduced to aristolactams or rearranged to 7-hydroxyaristolactams via Bamberger rearrangement. The intermediates (eg aristolactam-nitrenium) were regarded as responsible for the carcinogenicity of AAs (Fig 2). LC-MS and LC-MS-MS were applied to the analyses of a series of positional isomers of hydroxyaristolactams in both in vitro and in vivo samples. Metabolite identification provided important information for investigating mechanisms of AA toxicity and DNA formation. Prolonged exposure of AAs in rats was associated with development of renal disorder. Renal tubular atrophy and interstitial fibrosis were observed in rats dosed with up to 10 mg/kg/day of AAs for one month. In addition, AA-DNA adducts were detected in the rat kidney tissue.

The LC-MS method was applied to identify and

quantify the AA-DNA adducts isolated from kidney and liver tissues of AA-dosed rats.² The deoxycytidine adduct of AA (dC-AA), the deoxyadenosine-AA adduct (dA-AA), and deoxyguanosine adduct (dG-AA) were detected and quantified in the tissues of rats with a single oral dose AA (up to 30 mg/kg of body weight). The amount of AA-DNA adducts found in the rats correlated well with the dosage.

Furthermore, a specific method using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) was developed and applied to the determination of 7-(deoxyadenosine-*N*⁶-yl) aristolactam I (dA-AAI) in exfoliated urothelial cells of AA-dosed rats.³ After the isolation from urine samples, DNA in urothelial cells were subjected to enzymatic digestion and solid-phase extraction on a C18 Sep-Pak cartridge for the enrichment of DNA adducts. The sample extracts were analysed by reverse-phase UPLC-MS/MS with electrospray ionisation in positive ion mode. The quantification of the DNA-AA adduct was performed by using multiple reaction monitoring with reserpine as internal standard. The method was accurate and precise with a detection limit of 1 ng/ml, which allowed the detection of traces of dA-AAI in exfoliated urothelial cells (Fig 3).

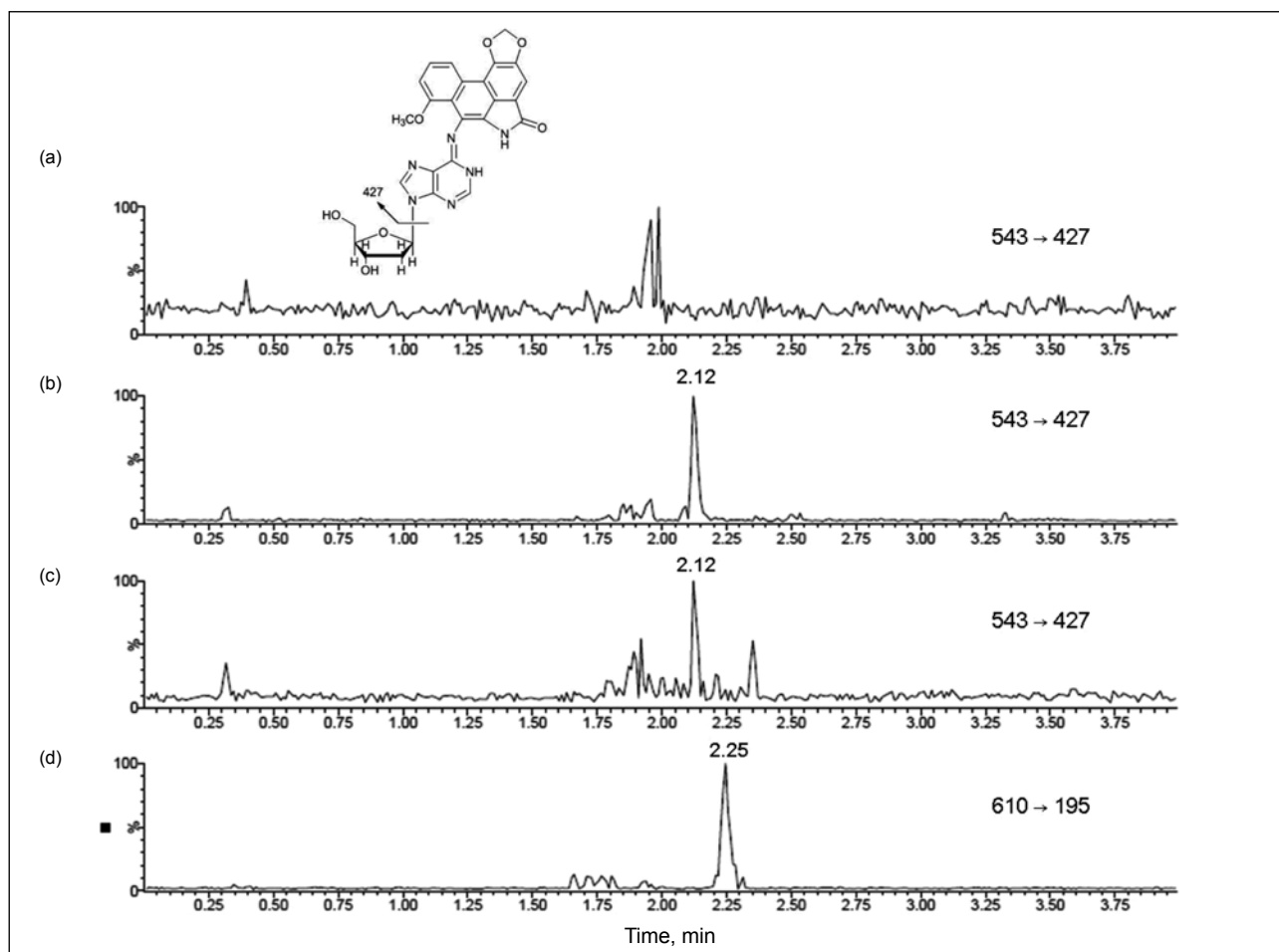


Fig 3. LC-MS/MS chromatograms for the analysis of dA-AAI (2.12 min) in (a) blank sample matrix, (b) spiked in blank sample matrix at a concentration of 60 ng/ml, and (c) exfoliated urothelial cells in urine collected from aristolochic acid-dosed rats. (d) Reserpine (2.25min) was used as internal standard at a concentration of 10 ng/ml.

In addition, a sensitive and rapid method entailing high-performance liquid chromatography with fluorescence detection (HPLC-FLD) was developed.^{4,5} The HPLC-FLD method enabled validation of the method and provided an alternative approach for analysis of AAs in Chinese medicinal herbs,⁴ as well as biological matrices.⁵

Discussion

Aristolochic acid nephropathy is associated with the prolonged exposure to nephrotoxic and carcinogenic AAs. DNA adducts induced by AAs have been proven to be critical biomarkers for AA-associated diseases. Metabolic activation of AAs produces reactive aristolactam-nitrenium ion intermediates. Electrophilic attack of the aristolactam-nitrenium ion via its C7 position on the exocyclic amino group in the purine bases leads to the formation of the DNA adducts.

Quantitative analyses of *in vivo*- and *in vitro*-formed DNA-AA complexes by the LC-MS method were performed with various quantification procedures. The LC-MS/MS method could provide specific and absolute quantification of DNA adducts in rat kidney tissue² and urine samples³ by combining the advantages of LC and multiple reaction monitoring. From rats dosed with AAs, adducts of AAs and DNA were detected in the biological samples, and dA-AAI was detected in urine. Nonetheless, a large volume of rat urine sample was needed for the urine analysis because the DNA-AA adduct levels generated by AA induction were relatively low in urine.

Although the sensitivity of urine samples in detecting the DNA adducts was low, the LC-MS/MS method can be an attractive alternative to the conventional ³²P-post-labelling assay for clinical diagnosis of kidney disease associated with AA poisoning, so long as its sensitivity was improved. In particular, a successful urine sample analysis for DNA adducts can provide the diagnosis without a biopsy. Further validation for its applicability to analyse human samples is warranted.

Because AAs can form covalent DNA adducts through

metabolic activation, the detection and characterisation of DNA-AA adducts is essential for the monitoring of exposure to AAs and for the investigation of their mutagenic and carcinogenic potential. The DNA adducts may thus serve as biomarkers of poisoning and can be used to confirm previous exposure. Therefore, the LC-MS/MS method for quantification of dA-AAI in exfoliated urothelial cells in urine of AA-dosed rats may be of clinical significance for diagnosing and monitoring AAs-associated disease in human. The detection of the DNA adduct in exfoliated urothelial cells is non-invasive and convenient. This method could also be applied to other DNA-AA adducts by monitoring the various multiple reaction transitions by tandem mass spectrometric analysis. Application of metabolomic approaches are recommended for analysing large amounts of animal data for detection of potential biomarkers associated with AA toxicity.

Acknowledgement

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