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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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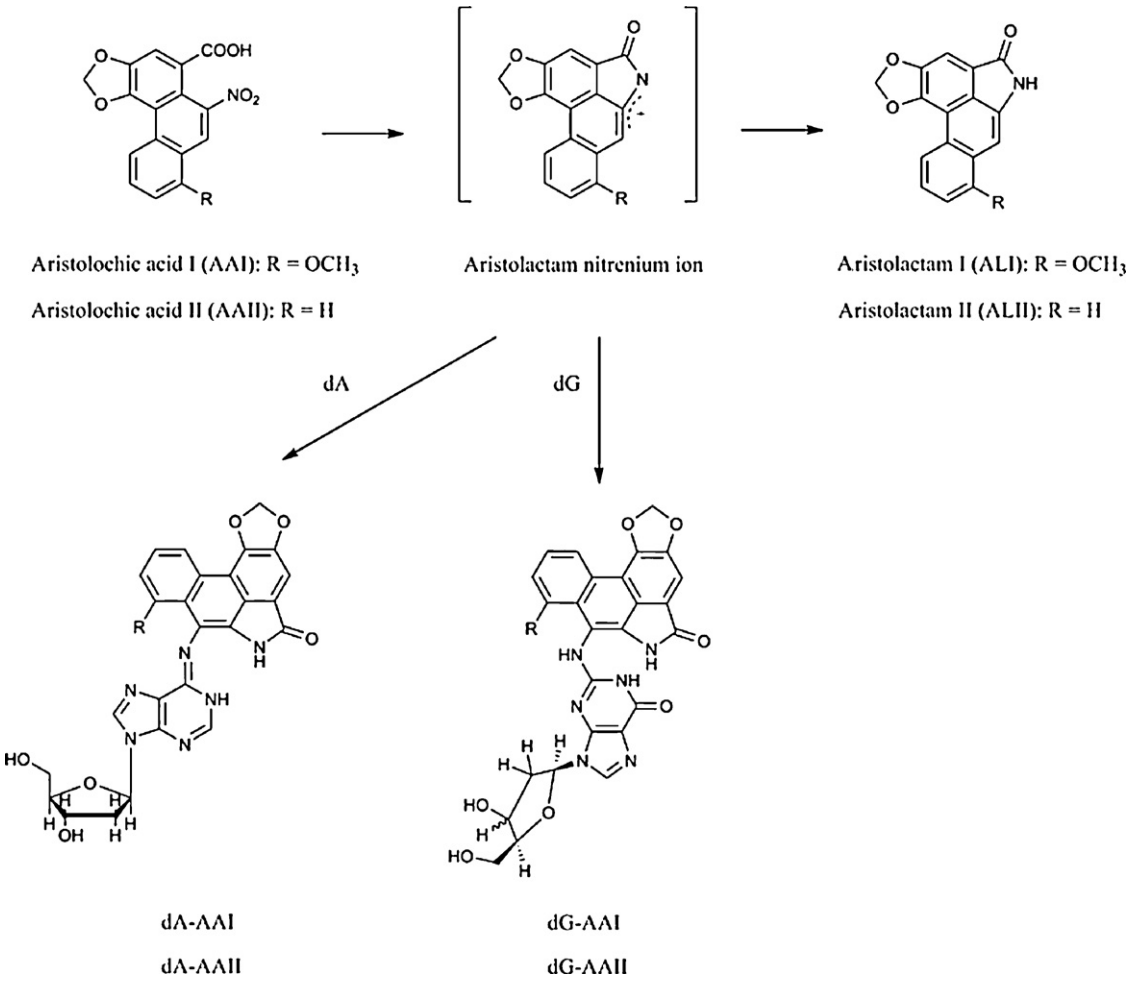

Fig. 1. Metabolic activation and DNA adduct formation of aristolochic acid I (AAI, R = OCH_3) and II (AAII, R = H).

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

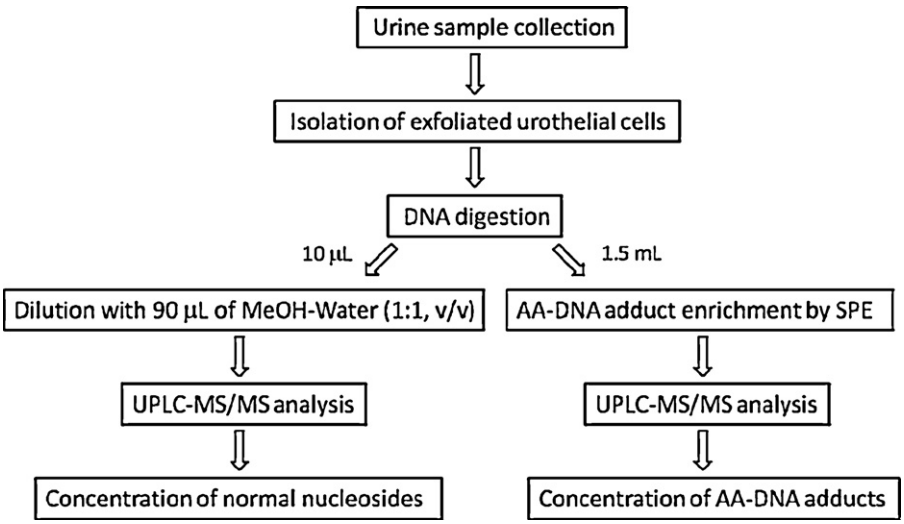

Fig. 2. Schematic sample preparation and quantification of AA–DNA adducts by UPLC–MS/MS.

sodium salt AAI-Na by reaction with sodium hydroxide and then suspended in 5 ml of 50 mM potassium phosphate buffer, pH 5.8. After the addition of 40 μmol of dA and 50 mg of zinc powder, the reaction was initiated by incubation at 37 °C for 16 h in the dark and stopped at 4 °C. The resulted reaction mixture was centrifuged to remove the zinc powder. The supernatant was lyophilized and re-dissolved in methanol for further purification by silica gel thin-layer chromatography. Chromatograms were developed in DEE followed by a mixture of DEE–MeOH–$NH_3{\cdot}H_2O$ (25:3:1, v/v/v). The purified dA-AAI was characterized by high-resolution mass spectrometry and fluorescence spectroscopy.

2.3. Animal experiment and urine collection

Animal experiment was conducted according to the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. The procedures were approved by the department of health, the government of the Hong Kong Special Administrative Region, China. Ten male Sprague–Dawley rats weighing 200–220 g were used in this study and kept in a room with controlled temperature, humidity and light/dark cycle. The rats were divided into two groups with each containing five rats, placed in metabolism cages and allowed to acclimatize to ambient conditions for five days prior to dosing. The dosed group was given an oral dosage of 10 mg/kg/day of AAI in 10 mM $NaHCO_3$ solution for one month, while the control group was treated with 1 ml of the dosing vehicle. Rat urine samples were collected for 24 h each day for the control and dosed groups, respectively, and kept at 4 °C. Body weight and urine volume of individual rats were recorded throughout the study.

2.4. Isolation of exfoliated urothelial cells

The entire pooled rat urine samples of approximate 3 l each were filtered through a double layer of cheesecloth followed by a 60-μm filter membrane and centrifuged at 400 × *g* for 10 min at 4 °C. The upper urine was discarded and the resulted pellet was washed three times by re-suspension in sucrose buffer and subsequent centrifugations. The sucrose buffer consisting of 0.25 M sucrose, 1.8 mM calcium chloride, 25 mM potassium chloride and 50 mM Tris–HCl (pH 7.5) was found to be able to maintain the good morphology of cells while removing the majority of contaminant crystals [26].

2.5. DNA digestion and adduct enrichment

A schematic illustration of sample preparation and analysis is shown in Fig. 2. As described previously [19,21], 0.7 ml of 5 mM sodium chloride in 0.01 M Tris buffer (pH 7.5), 30 μl of 1 mg/ml DNase I, 70 μl of 0.01 M magnesium chloride in 0.01 M Tris buffer (pH 7.0) were added to the washed pellet that was lysed by three freeze/thaw cycles, and incubated at 37 °C for 1 h. After that, 0.8 ml of 0.2 M Tris buffer (pH 9.0) and 30 μl of 1 unit/ml phosphodiesterase I were added and incubated for additional 48 h, followed by the addition of 22 μl of 3.5 unit/ml alkaline phosphatase and another 24-h incubation at 37 °C.

Due to the low level of AA–DNA adduct in the complex digested DNA sample, sample clean-up and DNA adduct enrichment prior to the UPLC–MS analysis were necessary. After reserving 10 μl of the digested solution for the determination of normal nucleosides, the rest extract (approximately 1.5 ml) was subjected to solid-phase extraction (SPE). A Waters C_{18} Sep-Pak cartridge was connected to a vacuum manifold, conditioned with 3 ml of methanol followed by 3 ml of water. The digested DNA solution was then applied to the column, which was sequentially washed with 3 ml of water, 3 ml of methanol/water (5:95, v/v), and 3 ml of methanol. The methanol fraction containing AA–DNA adducts was collected and evaporated to dryness under a stream of nitrogen at 37 °C. The obtained residue was reconstituted in 100 μl methanol for UPLC–MS analysis.

2.6. UPLC condition

UPLC analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent manager, an autosampler and interfaced to an electrospray ionization (ESI) source. Chromatographic separation was achieved on a Waters Acquity UPLC bridged ethyl hybrid (BEH) C18 column (2.1 mm × 50 mm i.d., 1.7 μm particle size). The mobile phase consisted of water (A) and acetonitrile (B). The gradient elution was programmed as follows: 0–1 min, 5% B; 1–2 min, 5–80% B; 2–2.2 min, 80–95% B; 2.2–3 min, 95% B; 3–3.1 min, 95–5% B, 3.1–4 min, 5% B. The mobile phase flow rate was maintained at 400 μl/min and the column temperature was kept at 25 °C. The injection volume was 10 μl.

2.7. Mass spectrometry analysis

MS/MS analysis was carried out on a Waters triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an

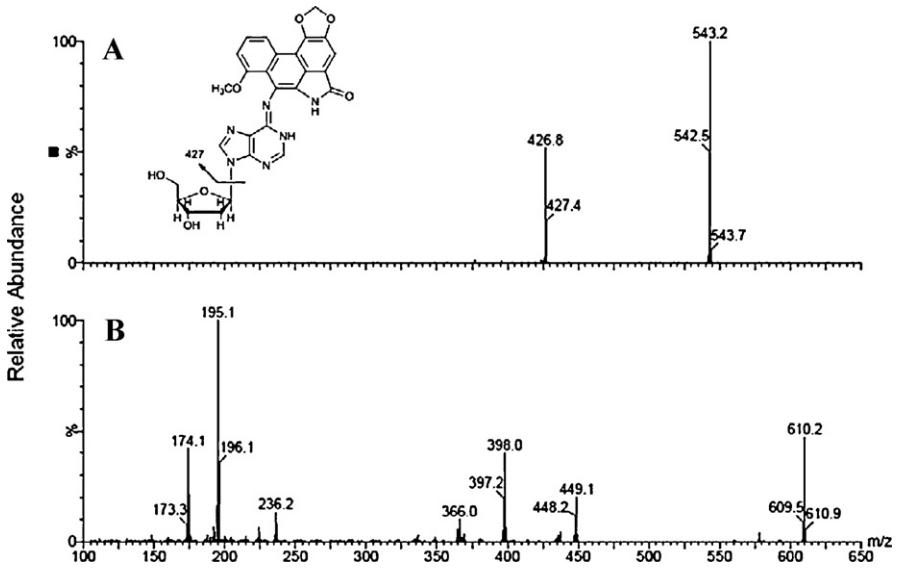

Fig. 3. Product ion spectra of dA-AAI and internal standard reserpine.

electrospray ionization source in positive ionization mode. The conditions used for ionization source were set as follows: capillary voltage of 3.0 kV, source temperature of 140 °C, desolvation temperature of 360 °C, desolvation gas flow of 600 L/h. Quantitation was performed in multiple reaction monitoring (MRM) mode and the conditions were optimized during direct infusion. For dA-AAI, the cone voltage and collision energy were set as 90 V and 23 eV, respectively. For the internal standard, cone voltage of 40 V and collision energy of 30 eV were applied. The dwell time was 0.05 s. The cone and collision gas flow were set as 20 L/h and 0.2 ml/min, respectively. Data analysis and quantitation were performed by using the Waters MassLync software.

2.8. Quantitative analysis of dA-AAI in exfoliated urothelial cells

Accurately weighed dA-AAI and internal standard were each dissolved in methanol and methanol/0.1% formic acid (1:1, v/v) to prepare the stock solutions. Urine samples collected from five control rats were processed as described above and used as blank sample matrix. A set of dA-AAI solution at seven concentration levels was prepared by serial dilutions of the stock solution with blank sample matrix. Each solution was spiked with 10 ng/ml reserpine as internal standard. Three replicate injections were made for each calibration level. The calibration curve was established by plotting peak area ratios of dA-AAI to internal standard versus the known dA-AAI concentrations. Linear regression data derived from the average of all three plots enabled the quantification of dA-AAI in exfoliated urothelial cells. Generally, the concentration of dA-AAI adduct was expressed as adducts per 10^9 normal nucleotides, the concentration of unmodified nucleosides was therefore determined by diluting 10 μl of the digested DNA samples with 90 μl methanol/water (1:1, v/v) for UPLC–MS analysis.

3. Results and discussion

3.1. Selection of internal standard

Due to the complexity of sample matrix, it was necessary to use internal standard for the correction of losses during the sample preparation and instrumental analysis. Since no commercialized stable isotope-labeled dA-AAI was available, reserpine was used as internal standard. Reserpine was an appropriate internal standard for the study because it was a synthetic chemical and thus could not be interfered by the complex sample matrix. From the MS/MS spectra of dA-AAI and reserpine shown in Fig. 3, it was observed that dA-AAI was clearly differentiated from reserpine with the obtained precursor ions and product ions.

3.2. Optimization of MS/MS analysis

MRM-based MS method development for the analyte of interest included the determination of the precursor ion, the optimization of the various lenses to maximize ion transmission, the selection of intense and specific parent–daughter transition, and the estimation of optimal cone voltage, collision energy and dwell time. Prior to the quantitative analysis, a 100 ng/ml standard solution of dA-AAI in methanol and reserpine in methanol/0.1% formic acid (1:1, v/v) was each infused directly into a tandem quadrupole mass spectrometer at a flow rate of 10 μl/min. Data acquisition was performed preliminary in full scan mode to choose an abundant precursor ion $[M+H]^+$. Under the daughter scan mode, the MRM transitions used for dA-AAI and internal standard detection were established, which corresponded to the intense and characteristic parent–daughter ion transitions. As shown in Fig. 3, a transition of 543→427 was selected for dA-AAI due to the characteristic loss of deoxyribose and a transition of 610→195 was chosen for the internal standard [18,27]. Three replicate infusions were performed for dA-AAI and reserpine for the optimizations of cone voltage and collision energy values as described in Section 2.

3.3. Method validation

Urine samples collected from five control rats were processed in the same way as those from the AA-dosed rats and used as blank sample matrix. A set of standard solutions of dA-AAI at different concentrations were prepared in blank sample matrix mixed with 10 ng/ml reserpine as internal standard. Plotting the peak area ratios of dA-AAI to internal standard against the concentrations of dA-AAI generated a calibration curve for dA-AAI with a coefficient better than 0.9995.

Sample preparation recovery of dA-AAI was evaluated by the analysis of blank sample matrix spiked with known amounts of dA-AAI. After clean-up and enrichment by SPE, the levels of dA-AAI were quantified by using the developed method, which were compared to the known amounts of spiked dA-AAI for obtaining

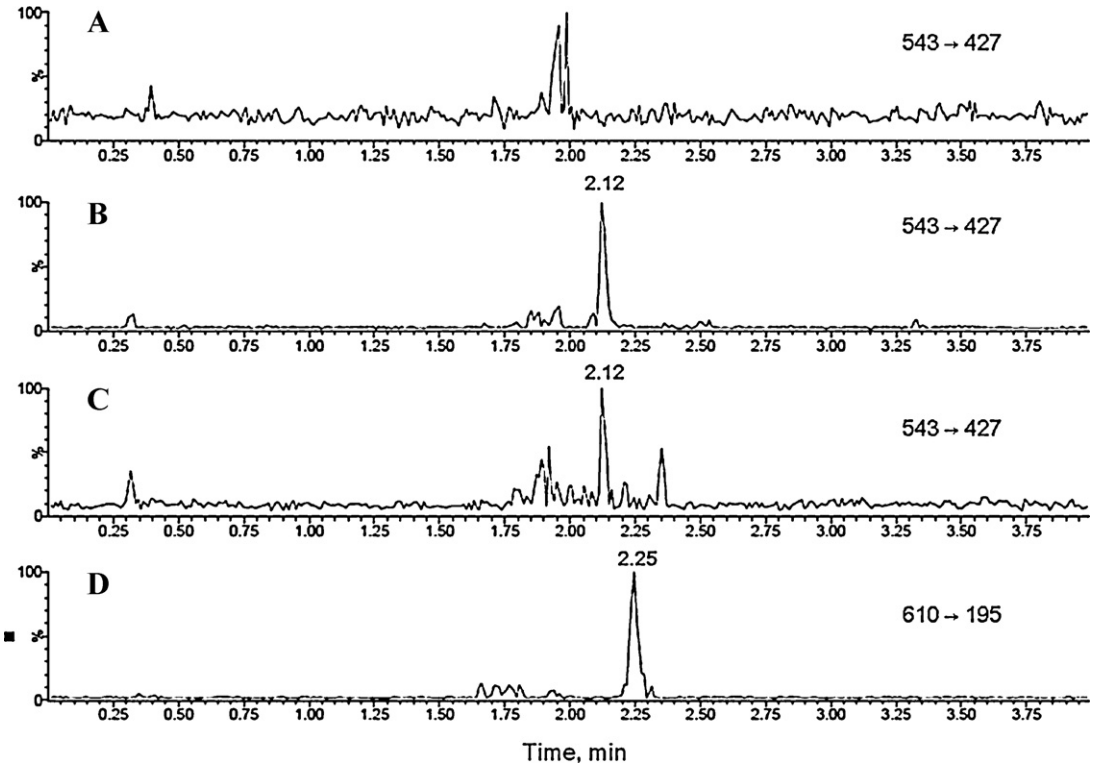

Fig. 4. MRM chromatograms obtained from the analysis of dA-AAI (2.12 min) in blank sample matrix (A), spiked in blank sample matrix at a concentration of 60 ng/ml (B), and in exfoliated urothelial cells collected from AA-dosed rats (C). Reserpine (2.25 min) was used as internal standard at a concentration of 10 ng/ml (D).

the sample preparation recovery data. The results indicated that the recovery was better than 90% for dA-AAI.

Spiked matrix samples were analyzed for evaluating the method accuracy and precision by using the developed method. The method accuracy expressed by relative error was determined by adding dA-AAI standard to blank sample matrix at low, medium and high concentrations (Table 1). Matrix blank was analyzed prior to the experiments. A typical MRM chromatograms obtained from the analysis of blank sample matrix and spiked matrix sample with dA-AAI at 60 ng/ml are shown in Fig. 4A and B, respectively. No significant matrix interferences were observed, indicating that the specificity of the method was acceptable. The obtained data showed that analytical error for dA-AAI was ranged from −4.0% to +2.8%. Intra-day precision was assessed from the replicate measurements ($n = 6$) of dA-AAI within one day and inter-day precision was evaluated from the analysis of the samples on three different days. As shown in Table 1, intra-day precisions (RSD) were within 3.1–4.5% for dA-AAI. Inter-day precisions (RSD) were within 4.0–6.1% for dA-AAI.

Limit of detection (LOD) and limit of quantification (LOQ) were obtained at levels giving signal-to-noise ratio (S/N) of 3 and 10, respectively. For dA-AAI, the LOD was determined to be 1 ng/ml and the LOQ was 4 ng/ml. This level of sensitivity is comparable with that of traditional ^{32}P-postlabeling method and HPLC-FLD.

Table 1
Accuracy and precision for the quantitative analysis of dA-AAI adduct.

Spiked level (ng/ml)	Found level (ng/ml)	R.S.D. (%)	Relative error (%)
Intra-day			
10	9.6 ± 0.43	4.5	−4.0
60	61.7 ± 2.37	3.8	2.8
140	140.5 ± 4.41	3.1	0.4
Inter-day			
10	10.2 ± 0.62	6.1	2.0
60	61.0 ± 2.43	4.0	1.7
140	141.4 ± 5.61	4.0	1.0

3.4. Quantitative analysis of dA-AAI in exfoliated urothelial cells

DNA adducts induced by AAs have been detected mainly in kidney and liver tissues of rodents treated with AAs and patients suffering from AAN and screened as an important biomarker for AAN. But the studies by Schmeiser et al. [28] showed that AA–DNA adducts were also detected in urinary tract tissues from AAN patients, although this organ is not directly targeted by AAs. Detection and accurate quantification of AA–DNA adducts in exfoliated urothelial cells, therefore, may be of clinical significance, because it can provide critical evidence to support clinical diagnosis of AA-related poisoning and disease and is non-invasive and convenient. Two groups of rats were used in this study. Each group contained five rats. One received the dosing vehicle as control samples and the other were dosed AAI at 10 mg/kg/day for one month. To obtain sufficient DNA for MS analysis, urine samples in each group were collected and pooled from five rats over one month. The DNA adduct in exfoliated urothelial cells were isolated, digested, enriched and clean-up by SPE, and analyzed by using the developed UPLC–MS/MS method. As shown in Fig. 4A and C, no dA-AAI was detected in the control samples, while 11.7 ± 0.62 ng/ml ($n = 3$) dA-AAI were detected in the exfoliated urothelial cells of AA-dosed rats. In comparison with the total amount of normal dA in digested DNA determined by UPLC–MS, dA-AAI level was detected at 2.1 ± 0.3 per 10^9 normal dA. This result was in accordance with the renal pathology study that showed tubular atrophy and interstitial fibrosis in kidney tissues of the AA-dosed rats. In contrast, the control group of rats without any detectable DNA adducts showed normal renal parenchyma.

The ^{32}P-postlabeling assay provided DNA adduct analysis by comparing the amount of the radioactive ^{32}P-labeled adducts with those of the ^{32}P-labeled normal nucleotides. However, inefficient

adduct labeling might impact the accuracy of adduct level to some extent. Moreover, the analytical facility with the capability of ^{32}P-postlabeling assay is rather limited due to the safety concerns on the use of large excess of radioactive γ-^{32}P labeled orthophosphate. The ^{32}P-postlabeling assay, for example, is currently unavailable in Hong Kong. As a result, all previous biological samples associated with AA-induced kidney disease were sent to laboratories in the U.S. or Europe for DNA adduct analysis and for the disease diagnosis. HPLC-FLD was an alternatively sensitive method for the quantification of DNA adducts by monitoring fluorescence intensity of varied AA–DNA adducts. Because their strong fluorescence was originated from the same fluorophore aristolactam, however, all of the AA–DNA adducts exhibit similar fluorescence excitation and emission spectra, which made the method less specific and extensively depend on the separation efficiency of HPLC. In contrast, the developed UPLC–MS/MS method could provide specific and absolute quantification of AA–DNA adducts in urine by combining the advantages of UPLC and MRM. In the current study, the detection of dA-AAI was clearly demonstrated in urine collected from rats dosed with AA. Similarly, the developed method could also be applied to the specific determination of other AA–DNA adducts by monitoring their characteristic MRM transitions from the tandem mass spectrometric analysis.

A large volume of rat urine sample was needed for the analysis probably due to the inadequate method sensitivity or the relatively low AA–DNA adduct levels in urine generated from the AA induction during the animal experiment. While the detection sensitivity for the DNA adduct needed to be improved further, the feasibility of using the UPLC–MS/MS method as an attractive alternative approach to the conventional assay was demonstrated. The developed method might have potential to serve for the clinical diagnosis of the kidney disease associated with AA poisoning with the improved method sensitivity. The successful urine sample analysis for DNA adduct could provide the diagnosis without the need of surgical procedure. The method should be further validated for its applicability for human sample analysis once the samples became available.

4. Conclusion

A novel and specific method using ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed and applied to the quantification of dA-AAI in exfoliated urothelial cells of AA-dosed rats. The analytes were detected in positive multiple reaction monitoring (MRM) mode by using reserpine as internal standard. The method provided good accuracy and precision with a detection limit of 1 ng/ml, which allowed the detection of dA-AAI in exfoliated urothelial cells at trace levels. The detection of the DNA adduct in exfoliated urothelial cells is non-invasive and convenient. Therefore, the developed method may be of clinical significance for diagnosing and monitoring AA-associated disease. Compared to other traditional methods such as ^{32}P-postlabelling and HPLC-FLD, UPLC–MS/MS analysis is more specific and rapid with an analytical time of less than 10 min. The developed method could also be applied to other AA–DNA adducts by monitoring their characteristic MRM transitions from the tandem mass spectrometric analysis.

Acknowledgements

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