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Aristolochic acid induced changes in the metabolic profile of rat urine

Wan Chan, Zongwei Cai *

Department of Chemistry, Hong Kong Baptist University, Kowloon, Hong Kong, SAR, China

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Abstract

Prolonged exposure to aristolochic acid (AA) has shown to pose rapid progressive renal fibrosis in Belgium women in a slimming regimen in the early 90 s. We hypothesize that changes in metabolic profile could have occurred before symptoms were observed, which may allow early treatment. In this study, metabonomics was used for toxicology study of AA in rats. Liquid chromatography coupled with a hybrid quadrupole time-of-flight mass spectrometry (Qq-TOF) was used for the analysis of endogenous metabolites in rat urine samples. The difference in metabolic profiles between the control and the dosed rats was well observed by the principal component analysis (PCA) of the MS data. Significant changes of two metabolite markers, kynurenic acid and hippuric acid, were detected in the rat urine samples. The identification of potential biomarkers was performed by high-resolution mass measurement and MS–MS analyses on a Qq-TOF. We believe that metabolic profiling may act as a preclinical protocol for AA exposure before symptoms are observed.

Keywords: Metabonomics; Nephrotoxin; Aristolochic acid; Biomarker; LC-MS

1. Introduction

Aristolochic acid (AA) is a mixture of structural-related nitrophenanthrene carboxylic acid derivatives (Fig. 1) found primarily in the genus *Aristolochia* [1]. During a slimming regimen in Belgium in the early 90 s, because of accidental replacement of *Stephania tetrandra* by AA-containing *Aristolochia fangchi*, about 100 cases of renal disease were reported. Some of the patients died and most of them required dialysis or kidney transplant [2]. DNA-AA adducts were detected in laboratory rodents after AA exposure [3–5] and in patients suffered from aristolochic acid nephropathy (AAN) [6–8].

Previous toxicology studies at molecular level focus mainly on the metabolites [9,10] and DNA adducts [3–8] formed. Metabonomics, which has been defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living organisms to pathophysiological stimulation or genetic modifications" [11], is emerging as a systematic approach to deal with changes in small molecular metabolites (MW <1000 Da) following toxic exposure, disease or variation in genetic function. By building up spectra library from certified patients suffered from hepatotoxicity, nephrotoxicity and cancers, metabonomics may act as a preclinical or clinical screening tool for potential patients.

High-field NMR has been used extensively for metabonomics [11–18]. However, with the advancement in hyphenated technologies such as LC-MS, it is a growing trend that metabonomic studies are turned to LC-MS based. It has been well demonstrated that LC-MS can act as a complement if not substitute for NMR for metabonomics studies [16–24]. While LC provides separation thus reducing spectra overlap and mass spectrometry being more sensitive when compared with NMR, it allows the detections of endogenous metabolites at a lower concentration. Identification of potential biomarker can be done by using the high-resolution mass and tandem MS capability of a Qq-TOF.

In the present study, a sensitive LC-MS method was developed for the analysis of endogenous metabolites in rat urine samples. Principal component analysis (PCA) of the chromatographic data well identified the control and the dosed rats based on the differences in their metabolic profiles. Potential biomarkers were identified from the high-resolution MS and MS–MS analyses.

* Corresponding author. Tel.: +852 34117070; fax: +852 34117348.
E-mail address: zwcai@hkbu.edu.hk (Z. Cai).

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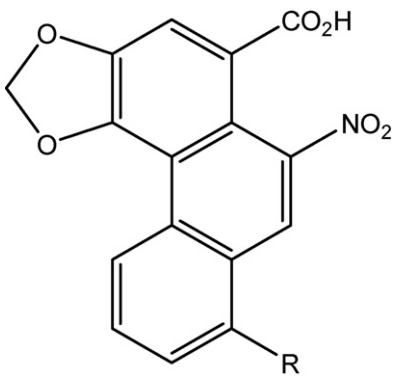

Aristolochic acid I: R=OCH_3
Aristolochic acid II: R=H

Fig. 1. Chemical structures of aristolochic I and aristolochic acid II.

2. Experimental

2.1. Chemicals

Aristolochic acid, mixture of aristolochic acid I and aristolochic acid II (approximately 1:1) was purchased from Acros (Morris Plains, NJ, USA). Renin substrate tetradecapeptide was purchased from Sigma (St. Louis, MO, USA). Formic acid and sodium hydrogen carbonate were obtained from Panreac (Barcelona, Spain). HPLC-grade acetonitrile was obtained from Tedia (Fairfield, OH, USA). Water was produced by a Milli-Q Ultrapure water system (Molsheim, France) with the water outlet operating at 18.2 MΩ.

2.2. Rat in vivo experiment and urine sample preparation

Male Sprague–Dawley rats weighing 200–220 g were used in this study and were acclimatized for 3 days prior to dosing. The animals were divided into two groups with each containing six rats. While the control group received 1 mL of the dosing vehicle, the dosed group received an oral dose of 10 mg/kg of AA in $NaHCO_3$ solution for 3 consecutive days. Rats were kept in metabolic cages in a controlled room with constant temperature (23 °C) and artificial dark and light cycles. Food and water was given *ad libitum* throughout the study. Urine samples from 8–24 h at 0–7 days post-dosing were collected and kept at −20 °C until sample pre-treatment. Body weight and urine volume of individual rats were recorded throughout the study.

Prior to the analysis, urine samples were thawed and centrifuged at 13,000 rpm for 3 min to aid settling of coarse material. Supernatant were removed, diluted at a ratio of 1:3 with Milli-Q water and vertex mixed for LC-MS analysis.

2.3. LC-ESI-MS analysis

2.3.1. HPLC system

Chromatographic separation of the endogenous metabolites was conducted on a HP 1100 HPLC system equipped with an auto-sampler (Palo Alto, CA, USA). Injection volume was 8 μL. A reverse-phase column (Symmetry C_{18}, 100 mm × 2.1 mm, 3.5 μm) (Waters, Milford, MA, USA), was used to separate the metabolites. The compartment of the auto-sampler was set at 4 °C throughout the analysis.

The mobile phase system used in this study consisted of 2 components, with component I being 0.1% formic acid (A), component II being acetonitrile (B). The solvent gradient was started from 10% (B) and held for 3 min, then programmed to 90% (B) in 12 min, and held for another 3 min before reconditioning, all at a flow rate of 300 μL/min.

2.3.2. MS system

Mass spectrometry was conducted on a Qq-TOF tandem mass spectrometer (API Q-STAR Pulsar i) (MDS Sciex, Toronto, Canada). Turbo ionspray parameters for positive ion mode ESI-MS were optimized as follows: ionspray voltage (IS) 4.5 kV, declustering potential I (DPI) 20 V, declustering potential II (DPII) 15 V, and focusing potential (FP) 70 V. The mass range chosen ranged from *m/z* 50 to 800. The IS, DPI, DPII, FP for negative ion mode ESI-MS were optimized as −4.3 kV, −20, −15 and −70 V, respectively. The ion source gas I (GSI), gas II (GSII), curtain gas (CUR), collision gas (CAD) and the temperature of GSII was set at 30, 15, 30, 3 and 350 °C, respectively.

Mass calibration of the TOF-MS was performed by infusion of 10 pmol/μL of renin substrate tetradecapeptide (*m/z* 110.0713 and 879.9723) in 1:1 acetonitrile:water +0.1% acetic acid at a flow rate of 10 μL/min. The calibration was done everyday before sample analysis in order to achieve good mass accuracy. Data acquisition and processing were performed on a personal computer with Analyst QS software (service pack 7) (MDS Sciex).

2.4. Data analysis of MS spectra

Acquired LC-MS data batches were subjected to the software of "Metabolomics Export Script" (MDS Sciex) for peak picking and noise reduction. TOF-MS data were processed by using the criteria as follows: minimum retention time 1 min, maximum retention time 25 min, noise threshold 50 counts/s, minimum spectra peak width 100 ppm, minimum retention time peak width 5 scan, maximum retention time peak width 150 scans, retention time tolerance 1.0 min, and maximum number of peaks 500. A list of the peak area of all components with their corresponding retention time and *m/z* as identifier was generated after sample normalization. The area list of individual peaks corresponding to endogenous metabolites was then exported for pattern recognition by PCA.

3. Results and discussion

3.1. Laboratory parameters from animal experiment

After the 7 days of experiment, a significant increase in urine volume was observed in the AA-dosed rats (Fig. 2A). The effect was most pronounced in day 5, probably significant biological effect had been induced on the rats by AA. The body weight of AA-dosed rats increased at a slower manner when compared

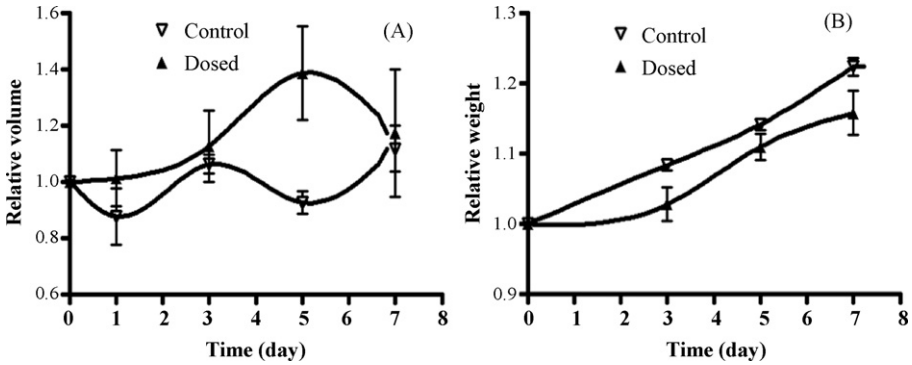

Fig. 2. Relative urine volume (A) and body weight (B) of the AA-dosed and control rats.

with that of the control ones (Fig. 2B). For the fact that both groups of rats were allowed free access to water and food, the result demonstrated that AA induced a decrease in growth rate in rats. Both the decline of Krebs cycle and abnormal reabsorption ability of renal tubules could be the causes for the decrease in growth in the AA-dosed rats [15].

3.2. Sample preparation and LC-MS analysis

Urine is a complex matrix containing a wide variety of acidic, neutral and basic compounds with high polarity. Sample pre-treatment by conventional methods such as solid-phase extraction or liquid–liquid extraction may results in loss of the metabolites with high polarity and high hydrophilicity. While metabonomics being a non-targeted analysis of the global system for changes in endogenous metabolites, sample pre-treatment may result in loss of potential biomarker. Thus, minimal sample preparation steps were employed in urine samples analyzed in order to avoid the loss of the endogenous metabolites. The urine samples were centrifuged and diluted prior to the direct injection into the reversed-phase HPLC column for the LC-MS analysis.

To minimize thermal degradation of the metabolites while waiting to be analyzed, the auto-sampler compartment was set at 4 °C throughout the analysis. It was however, inadequate time allowing the samples to get equilibrated to the pre-set temperature would introduce errors in which sample introduction volume in the first few samples indifferent from the proceeding ones. By repeating the analyses of the same urine sample starting at room temperature, it was found that a 50 min equilibrium period was required for achieving a low deviation in peak area (∼5%).

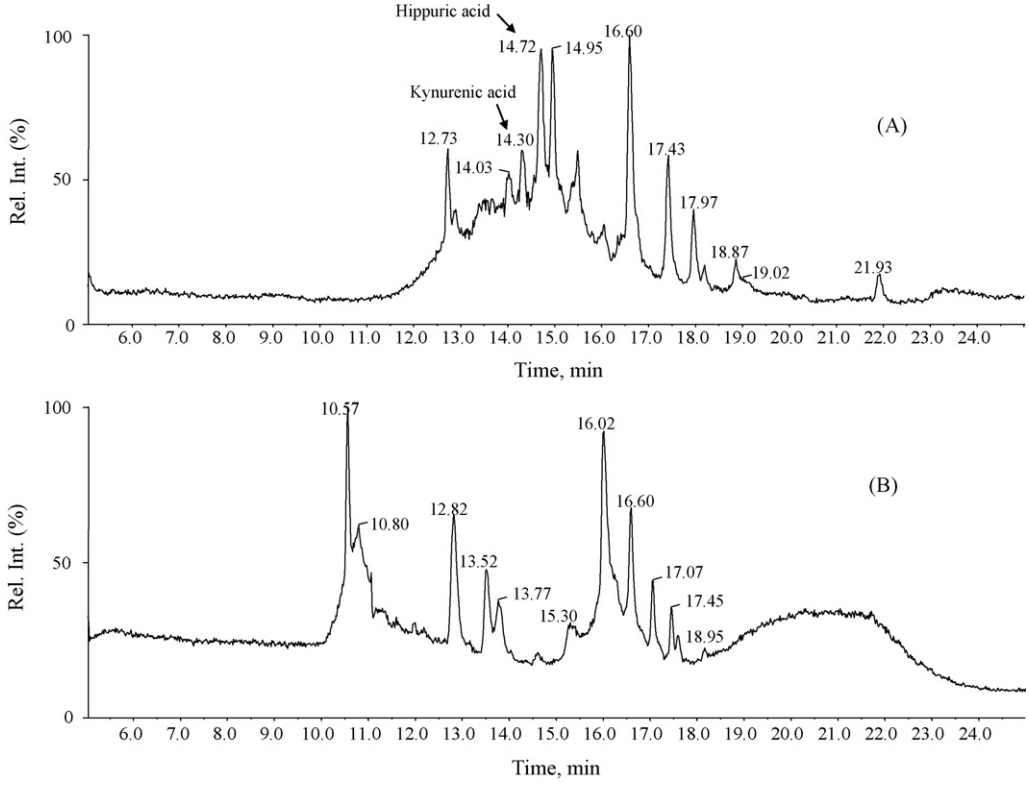

Fig. 3. Total ion chromatograms from the LC-MS analyses of rat urine samples by using positive (A) and negative (B) electrospray ionization.

High reproducibility is crucial for any analytical protocols, especially for metabonomics study which requires handling tens to hundreds of samples. Reproducibility of the chromatography and MS was determined from seven replicated analyses of the same urine sample. With hippuric acid as standard, the chromatographic retention time and peak area were measured with variation of 0.78 and 5.02% R.S.D., respectively, indicating that the LC-MS method was highly reproducible and could be used as a promising tool for metabonomic studies.

Both positive and negative ion modes of ESI-MS were used for the LC-MS analyses of the urine sample under the identical chromatographic conditions in order to obtain maximal information of the metabolites. Typical total ion chromatograms (TIC) in positive and negative ionization modes obtained from the LC-MS analysis of rat urine sample collected from the control animal are shown (Fig. 3).

3.3. Principal component analysis

Principal component analysis, a chemometric model which reduces matrix of data to their lowest dimension of the most significant factors, was used for analyzing the chromatographic data. Peak list generated by the "Metabolomics Export Script" from the positive LC-MS analyses was provided to the PCA program for pattern reorganization. The obtained PCA plot from the processing of data from the entire animal study is shown in Fig. 4, with the data corresponding to the dosed rats being enclosed in the ringed region. The rats subjected to the same dosing conditions located on the same trajectory group, indicating that they could have similar metabolic profiles. Clear differentiation of the control and the dosed rats were observed, indicating that there was significant difference

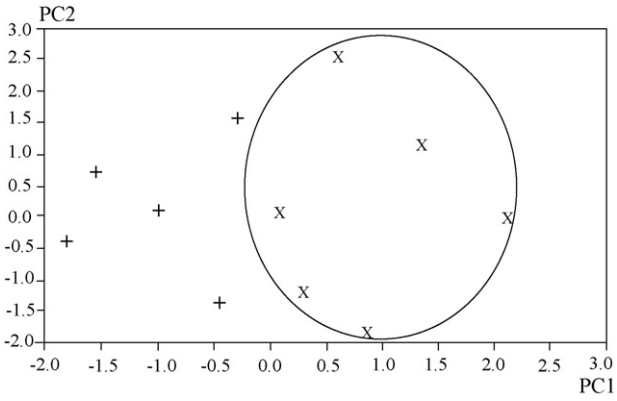

Fig. 4. PCA plot of the data set obtained from control and dosed (ringed) rats.

in the urine samples collected from the control and the dosed rats. Though being a preliminary work for the nephrotoxicology study, the obtained PCA data did show the difference between the control and dosed rats when the animal experiment and urine sample analysis by LC-MS were conducted in the same way, suggesting that the metabonomic study could be used to indicate whether the animal had been dosed with the toxic AA or not.

3.4. Biomarker identification

Base peak chromatogram (BPC), the chromatographic plot displaying the intensity of the most intense ion within a scan versus retention time, is considered as an efficient and convenient way for chromatogram comparison. A preliminary comparison of the BPC of the control and the dosed rats at 8–24 h (Fig. 5) showed marked difference. The metabolites eluting at 14.7, 18.0

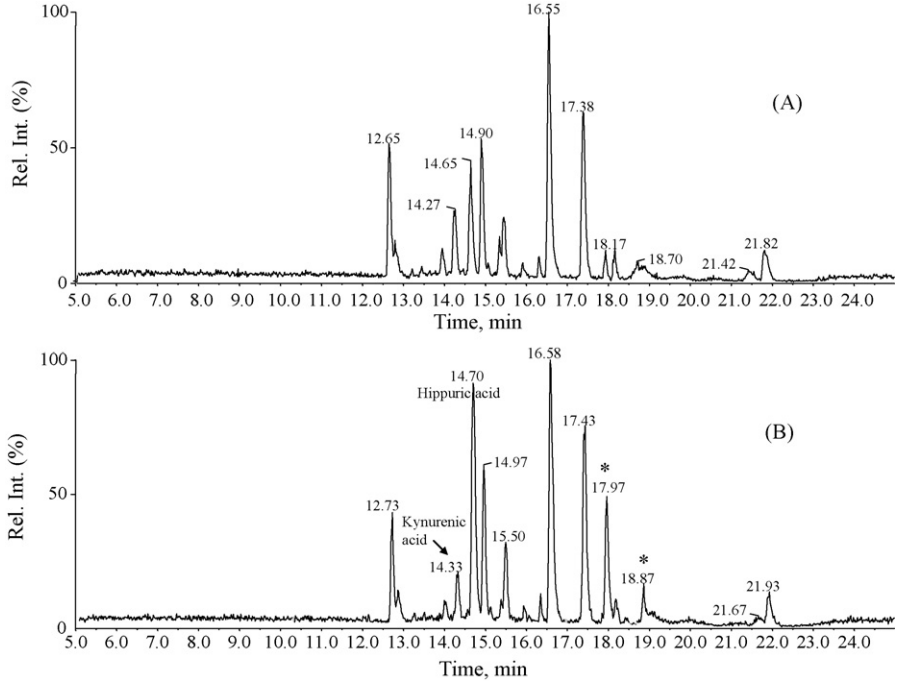

Fig. 5. Base peak chromatograms obtained from the positive ion LC-MS analyses of control (A) and dosed (B) rats.

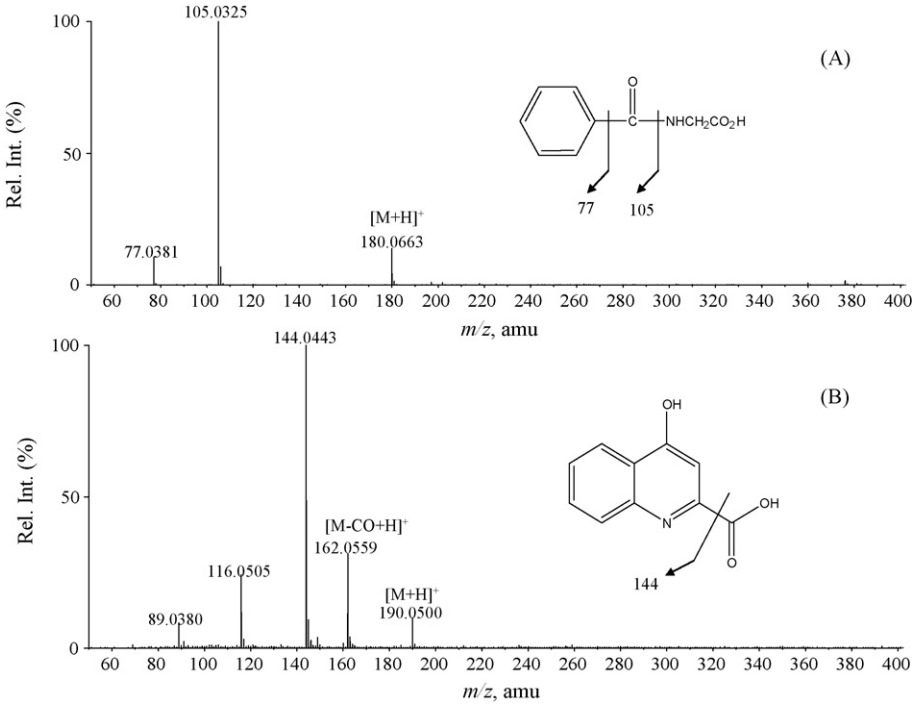

Fig. 6. MS–MS spectra of the $[M+H]^+$ ion of hippuric acid (A) and kynurenic acid (B) at *m/z* 180 and *m/z* 190, respectively.

and 18.9 min increased while a metabolite at 14.3 min reduced in relative concentration after dosing. MS spectra of the metabolites eluting at 14.3 min and 14.7 min showed $[M+H]^+$ ion peak at *m/z* 190.0516 and *m/z* 180.0663, respectively, together with their $[M+Na]^+$ ion peak.

Metabolite identification was confirmed by analyzing the MS–MS spectra of the $[M+H]^+$ ion (Fig. 6) and they were confirmed to be kynurenic acid (*m/z* 190.0504) and hippuric acid (*m/z* 180.0661) with a mass difference of 6.3 and 1.1 ppm, respectively. Similar changes in metabolic concentration were observed in previous LC-MS based metabonomics investigation of the model nephrotoxin mercury chloride [20]. Further studies are needed for the biomarker-related kidney disease caused by the AA.

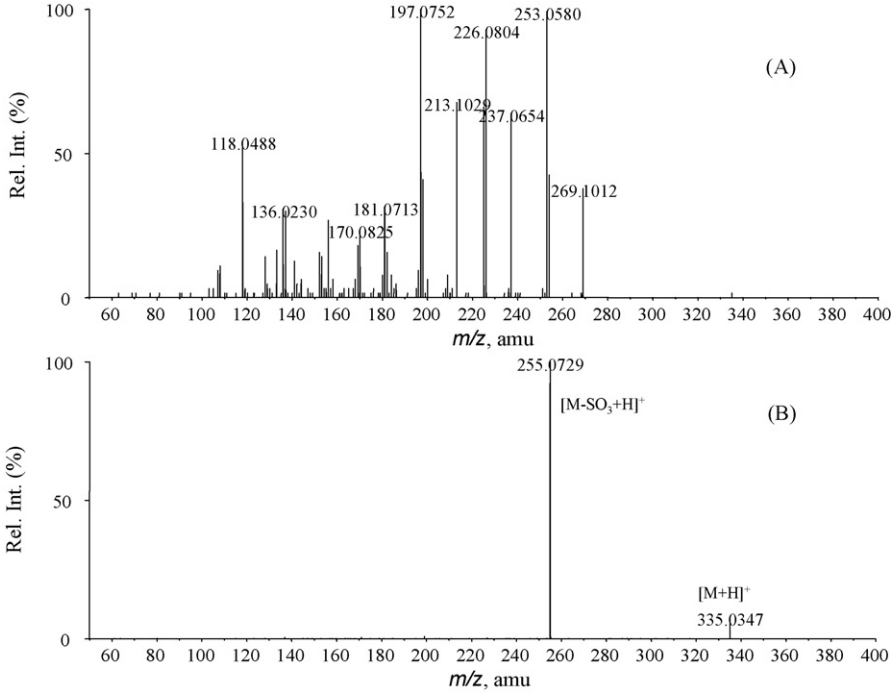

Fig. 7. MS–MS spectra of the $[M+H]^+$ ion of the potential biomarkers at *m/z* 269 (A) and *m/z* 335 (B).

Chen *et al.* reported the identification of some potential biomarkers as a consequence of AA exposure [23]. However, most of those "biomarkers" were identified solely based on *m/z* obtained from a low-resolution mass spectrometry while confirmation with authentic standards and MS–MS analysis was not performed. The identification of an unknown compound with only MS data is difficult, sometimes impossible even on a Qq-TOF in which the high-resolution MS analysis provides elemental composition and MS–MS experiments provides information for the fragmented ions, when the authentic standard is unavailable. Although the ESI is considered as a soft ionization technique, it is sometimes hard to determine whether an ion peak in the spectrum corresponds to a molecular ion or a fragment ion that may have been produced from source collision induced dissociation. The detection of $[M+H]^+$ ions in the LC-MS analysis of the rat urine samples was confirmed by the simultaneous observation of the $[M+Na]^+$ ion. When the sodiated adduct ion was not simultaneously observed for a suspected protonated molecular ion of the potential biomarkers, addition of volatile buffer such as ammonium acetate or ammonium formate was found helpful because $[M+NH_4]^+$ ion was detected from some metabolties, confirming the discovery of biomarker molecular ions.

It was challenging to identify the metabolites eluted at 18.0 and 18.9 min, which showed $[M+H]^+$ ions at *m/z* 269.0803 and *m/z* 335.0201, respectively, along with their $[M+Na]^+$ ion. The MS–MS spectra of these two potential biomarkers are shown in Fig. 7. Analyses of high-resolution mass spectrometry data and isotope patterns of the ion at *m/z* 335.0201 indicated that the compound might contain sulfur element. MS–MS analysis of the $[M+H]^+$ ion showed the intense peak of major fragment ion resulted from the neutral loss of a sulfate group in the metabolite molecule (Fig. 7B). Further investigations on these two potential biomarkers are needed for their structural elucidation.

4. Conclusion

A sensitive LC-MS method for metabonomic study of the nephrotoxin aristolochic acid was developed. The method allowed the discovery of potential biomarkers within complex sample matrix of urine. Biomarker identification among the potential endogenous metabolites was performed by the high-resolution mass measurement and the MS–MS analysis on a Qq-TOF. PCA of the data demonstrated the difference between the control and the dosed rats based on their metabolic profiles. The work of metabolic profiling in plasma samples is in progress.

Acknowledgements

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