FULL PAPER
THE ANALYST
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Identification and quantification of urinary benzo[*a*]pyrene and its metabolites from asphalt fume exposed mice by microflow LC coupled to hybrid quadrupole time-of-flight mass spectrometry

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Received 4th March 2003, Accepted 15th April 2003
First published as an Advance Article on the web 6th May 2003

Prolonged, extensive exposure to asphalt fume has been associated with several adverse health effects. Inhaled polycyclic aromatic hydrocarbons (PAHs) from asphalt fume exposure have been suspected of inducing such effects. In this study, a bioanalytical method was proposed and evaluated to identify and quantify benzo[*a*]pyrene and its hydroxy-metabolites. This method is based on coupling a microflow liquid chromatography (LC) to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometry (Q-TOFMS). In the experiment, thirty-two B6C3F1 mice were exposed to asphalt fume in a whole body inhalation chamber for 10 days (4 h day^{-1}) and twelve other mice were used as controls. The asphalt fume was generated at 180 °C and the concentrations in the animal exposure chamber ranged 175–182 mg m^{-3}. Benzo[*a*]pyrene and its metabolites of 3-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dihydrodiol(±), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), and benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) in the urine of asphalt fume exposed mice were identified and found at 3.18 ng 100 mL^{-1}, 31.36 ng 100 mL^{-1}, 11.56 ng 100 mL^{-1}, 54.92 ng 100 mL^{-1}, and 45.23 ng 100 mL^{-1} respectively. The results revealed that the urinary benzo[*a*]pyrene and its hydroxy-metabolites from exposed mice were at significantly higher levels ($p < 0.001$) than those from the control groups. Compared with several other technologies such as HPLC-UV and HPLC-fluorescence, the new method is more sensitive and selective, and it can also provide additional useful information on the structures of the metabolites. Hence, this method can be used to perform the assessment and to study the mechanisms of the adverse health effects. The fragmentation patterns established in this study can also be used to identify and quantify PAH metabolites in other biological fluids.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important class of toxicants that have been found in asphalt fume.[1,2] It is estimated that approximately 4,000 hot-mix asphalt facilities and 7,000 paving contractors employ nearly 300,000 workers in the United States.[3] Prolonged, extensive occupational exposure to asphalt fume may pose a risk to workers. Inhaled PAHs are the major health concern associated with asphalt fume exposure. It has been reported that road paving workers can be exposed to 0.1–2 mg m^{-3} bitumen fume, which can include 10–200 ng m^{-3} benzo[*a*]pyrene.[4] Data from studies with animals indicated that laboratory-generated roofing asphalt fume condensates were genotoxic[5] and produced skin tumors in mice.[6]

Asphalt is an extremely complex mixture.[5] It contains persistent PAHs and other organic compounds that exhibit a high accumulation potential in living systems.[7] Inhaled benzo[*a*]pyrene, a procarcinogenic PAH, had long been suspected of inducing cancer. It has been estimated that crude asphalt contains the most widely distributed class of potent carcinogens presented in the human environment.[8] Twenty epidemiological investigations on the cancer risk in asphalt road pavers and roofers invariably concluded that there were indications of increased risks for lung, stomach, and nonmelanoma skin cancers and for leukemia in roofer populations.[9] The relative risks for the same cancers in road pavers and highway maintenance workers were lower than those in roofers, probably because the roof asphalt fume generally has a higher temperature than the road asphalt fume. The exact mechanisms of the effects, however, remain unclear and further studies are required.[10,11]

The application of urinary metabolites as biomarkers has been growing in studying workplace exposure.[12] Urinary naphthols (1- and 2-naphthol) have been suggested as route biomarkers for airborne PAHs. In one study,[13] the urinary naphthols in 119 Japanese workers were reported. The urinary 1- and 2-naphthol levels were observed three and sevenfold times higher, respectively, among smokers than among nonsmokers. In another study,[14] a method for the determination of urinary PAH metabolites was used for individual risk assessment at a PAH-burdened workplace. In other works, the PAH exposure to coke plant workers during several consecutive days resulted in fairly constant individual urinary PAH metabolite profiles.[15] This study also indicated that there was a correlation between inhaled PAH and metabolites excreted. Mass relationships between inhaled PAH and metabolites excreted were observed to differ from one individual to another. In addition, a LC method with fluorescence detection was reported for the determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene in the urine of PAH-exposed workers.[16] The concentrations ranged 3–198 ng of 3-hydroxybenzo[*a*]pyrene g^{-1} of creatinine and 15–187 ng of 3-hydroxybenz[*a*]anthracence g^{-1} of creatinine in 19 workers engaged in the production of fireproof materials. The applications of LC-MS in analysis of workplace and environmental samples is increasing.[17–19] How-

 DOI: 10.1039/b302617p

ever, the studies on the identification and quantification of PAHs and their metabolites associated with occupational exposure using the advanced MS technology are still limited.[5]

The analytical challenge associated with developing a selective and reliable methodology for identification and quantification internal levels of PAHs and their metabolites are considerable. Because of the complexity of asphalt fume, it is difficult to establish a causal relationship between one toxicant and an adverse health effect or a dysfunction. Assessing occupational exposure to asphalt fume is especially problematic. Methodologies for evaluating a complex mixture are not well established. Currently, no specific adverse health response was established for exposure to asphalt fume; the hazards from such exposure are unclear.[6] In part, this may be because of a lack of suitable biomarkers that remain to be identified. Therefore, the specific aims of this study are (i) to develop a set of practical experimental procedures to characterize PAH metabolites based on a nanoflow LC-Q-TOFMS technology, (ii) to establish the fragmentation patterns to characterize the metabolites, and (iii) to identify and quantify urinary benzo[*a*-]pyrene and its hydroxy-metabolites from asphalt fume exposed mice.

Materials and methods

Chemicals

Reference metabolites of 3-hydroxybenzo[*a*]pyrene, benzo[*a*]-pyrene-7,8-dihydrodiol(±) benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), and benzo[*a*]pyrene-7,8,9,10-tetrahydro-tetrol(±) were purchased from NCI Chemical Carcinogen Reference Standard Repository (Kansas, MI, USA). 6-Hydroxychrysene (ring $^{13}C_6$, 98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). QTM PAH Mix reference material was purchased from Supelco (Bellefonte, PA, USA). ES Tuning Mix was purchased from Agilent Technologies (Wilmington, DE, USA). Reagent grade dichloromethane (CH_2Cl_2, 99.9+%), acetonitrile, hexane and perdeuterated anthracene were purchased from Aldrich (Milwaukee, WI, USA). The test asphalt was the type used by the paving industry (Hot Performance Grade Asphalt PG 64-22).

Analytical supplies and instruments

Polytetrafluoroethylene (PTFE) filters (37 mm, 0.45 μm pore size) and XAD-2 traps [treated with 2-(hydroxymethyl) piperidine] were purchased from SKC (Eighty Four, PA, USA). Solid phase extraction cartridges of EnvirElut PAH (500 ng/2.8 mL) were purchased from Varian (Harbor City, CA, USA). PTFE tubes (30 mL) and glass tubes (10 mL) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Target DP vials (1.5 mm with 200 μL inserts) were obtained from Alltech Associates, Inc. (Deerfield, IL, USA). Extraction of asphalt fume from collection media was performed by ultrasonic extraction (FS-220, Ultrasonic power 320W, Fisher Scientific, Fairlawn, NJ, USA). A BenchMate™ II Workstation (Solid-Phase Extractor, Zymark, Hopkinton, MA, USA) and syringe filter (25 mm, 0.2 μm pore size) specially designed for HPLC samples (Gelman Sciences, Ann Arbor, MI, USA) were employed to perform the purification and filtration of the sample solutions. Extracts were reduced under a nitrogen stream using a TurboVap® LV evaporator (Zymark). Liquid nitrogen, high purity helium, and argon were purchased from Butler Gas Products Co. (Mckees Rocks, PA, USA), and used as GC-MS (Hewlett Packard, Wilmington, DE, USA) and hybrid Q-TOFMS II (Micromass Inc. Beverly, MA, USA) carrier gases. The GC column was HP-5 MS, 95% dimethylpolysiloxane, Nonpolar, 30 m length, 0.53 mm id (Hewlett Packard), and the microflow LC column used was Nucleosil C_{18} PAH, 5 μm, 1000 μm id (LC Packings, San Francisco, CA, USA). Forty-eight female B6C3F1 mice (6–8 weeks) were purchased from Taconic (Germantown, NY, USA).

Animal inhalation exposure and asphalt fume sample collection

Forty-eight B6C3F1 mice were certified to be pathogen free and post examined by a veterinarian. The mice were housed in pairs in a metabolism cage during a two week acclimatization period. Thirty two test mice received the asphalt fume exposure in a whole body inhalation chamber for 4 h day^{-1} over 10 days. The generation of asphalt fume was conducted in the National Institute for Occupational Safety Health inhalation facility. A dynamic asphalt fume generation system (Heritage Research Group, Indianopolis, IN) was modified to provide the asphalt fume. A computer control system was incorporated into the system to improve performance and to simplify operation. The test asphalt was representative of the formulation used throughout the mid-western United States. For fume generation, the asphalt was initially preheated in an oven to 200 °C, and then pumped into a large bitumen kettle that maintained the asphalt temperature. The heated asphalt was then transferred to the generator where fume was generated above the asphalt surface as the asphalt flowed over a heated generator plate. Air, heated to 180 °C, passed over the upper surface of the asphalt and transported the volatile fraction to the animal exposure chamber *via* a short heated transfer line. The test chamber was able to hold 16 mice for the exposure period. The sampling train for the fume consisted of a PTFE filter collection of particles, followed by a second stage, an XAD-2 tube [treated with 2-(hydroxymethyl) piperidine] that trapped the vapor fraction from the influent (1.0 L min^{-1}) vapor. Fume samples were collected immediately post the generator and at entry into the exposure chamber. The test animals were sacrificed immediately after the last exposure.

Urine sample collection and preparation

The urine of exposed mice was collected into a polypropylene tube every 24 h through a mouse metabolism cage and was frozen immediately at −20 °C. Control urine from non-exposed mice was collected for 10 days. A few processing steps were performed to manipulate the urine samples into a form suitable for analysis. The procedures involved hydrolysis of conjugated benzo[*a*]pyrene metabolites, and purifying them from interferences before determining by microflow LC-Q-TOFMS. Briefly, a 15 mL volume of urine was added 15 μL ß-glucuronidase/arylsulfatase and the pH was adjusted to 5.0 with 1 M hydrochloric acid. The sample solution was incubated for 16 h at 37 °C. An internal standard 6-hydroxychrysene (ring $^{13}C_6$) was added to the sample at a final concentration of 10 ng μL^{-1}. The urine purification stage was performed using solid phase extraction to remove impurities that could interfere in target analytes detection. In operation, the urine was transferred to a smooth-walled test tube. This tube was loaded onto the sample position of a BenchMate™ II Workstation. The EnvirElut PAH cartridge (Varian) was conditioned with a solution of 80% water + 20% acetonitrile. After the sample was loaded, the cartridge was washed with water and dried for 1 min by air. Then, the target analytes were slowly eluted and collected with diethyl ether. Finally, the extract solutions were concentrated by reducing the solvent of the sample under a nitrogen stream using a TurboVap® LV Evaporator. Control urine samples were prepared using the entire analytical procedure as well as the same reagents as those used for treating asphalt fume urinary samples. The recovery tests were carried out using spiked

solutions of benzo[*a*]pyrene, 3-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dihydrodiol(±), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol (±), and 6-hydroxychrysene (as an internal standard, ring $^{13}C_6$). The yield of recovery was determined by micoflow LC-Q-TOFMS.

Asphalt fume in mice exposure chamber

The concentrations of asphalt fume in the mice exposure chamber were determined by GC-MS. The fume sample preparation involved desorption, filtration, and preconcentration. PTFE filter or XAD-2 trapping absorbant samples were transferred to separate PTFE tubes, and dichloromethane/hexane (50/50) was added. Ultrasonic extraction was performed using an FS-220 Ultrasonicator (320W). After desorption of asphalt fume from the collection medium, the extract was filtered using a BenchMate™ II Workstation, which was programmed to perform procedures automatically. Preconcentration was performed under nitrogen using TurboVap® LV Evaporator. Sample extracts were reconstituted with dichloromethane. Concentrations of asphalt fume in the animal exposure chamber were determined by GC-MS. The instrument was calibrated by a mixture of 16 reference PAHs, and perdeuterium anthracene was used as internal standard. The total ion chromatograms were acquired with a 3 min solvent delay. Separation was performed on a HP-5 MSD capillary column (30 m length, 0.53 mm id) with a temperature program from 50 °C to 310 °C at an increasing rate of 5 °C min^{-1}. Calibration curves were developed with 5 point measurements. The recovery of the asphalt fume was evaluated by adding stable isotope perdeuterated anthracene in the samples and determined by GC-MS.

Microflow LC-Q-TOFMS

The electrospray ionization combining with the second dimension of TOFMS-MS is particularly useful for selected PAH and its metabolite detection. The merit of coupling a microflow LC to a hybrid Q-TOFMS system is that it offers highly specific accuracy mass measurement (~5ppm) and resolution (~10,000) for developing a sensitive and selective bioanalytical method. A microflow LC column used was Nucleosil C_{18} PAH, 5 µm, 1000-µm id. A gradient elution (15 µL min^{-1}) profile was generated with solvents (A) 98% water + 2% acetonitrile + 0.1% formic acid, and (B) 90% acetonitrile + 10% water + 0.1% formic acid. The separation was conducted from mobile phase contained 95% (A) at 0–5 min to 85% (A) at 5–10 min, and then to 75% (A) at 10–15 min, to 60% (A) at 15–20 min, to 30% (A) at 20–25 min, to 5% (A) at 30 min. After running 30 min, the mobile phase went to 95% (A) at 31–55 min. The operational features in Q-TOFMS system consist of a standard Z-spray source fitted with a heated nebulizer probe. Electrospray positive ionization was used for the analysis. The source temperature was set at 90 °C. The instrument was calibrated with a multi-point calibration technique using selected fragment ions that resulted from the collision-induced dissociation of [Glu]-fibrinopeptide B (Sigma Chemical Co.). The limits of detection was 20 fmol of [Glu]-fibrinopeptide B determined by the signal to noise greater than 3:1. With the flow rate of 5 µL min^{-1}, the limit of quantitation (LOQ) was determined to be 30 fmol. The linear dynamic range was around 3 orders of magnitude (0.1–40 pmol). In all cases, calibration regression coefficients were between 0.95 and 0.99. The precision was determined by repeating injection 5 times (RSD < 15). Once optimized, operating parameters were maintained at a constant condition throughout the experiment.

Results and discussion

Characterization of benzo[*a*]pyrene and its hydroxy-metabolites

Benzo[*a*]pyrene and its hydroxy-metabolites were characterized by microflow LC-Q-TOFMS. A gradient elution from a PAH column was optimized to separate the target analytes as described in the Experimental section. The detection limits of reference standards were ranged 40–60 pg based on the signal to noise ratio greater than 3:1. The system provides proper precursor ion resolution by virtue of their double focusing properties, which can be used to obtain MS-MS information from precursor ions of the same nominal mass. In principle, the competing ionization mechanism in positive electrospray was protonated form $[M + H]^+$.[20] With the experimental conditions as described above, the greatest ion intensities in these studies were observed *via* proton transfer. During collision-induced dissociation, the molecular ion under investigation was collided with a collision gas and acquired internal energy, which lead to its decomposition into product ions and generated a specific TOFMS-MS spectrum.

The precursor ions of *m*/*z* 321 $(C_{20}H_{16}O_4 + H)^+$, *m*/*z* 303 $(C_{20}H_{14}O_3 + H)^+$, *m*/*z* 287 $(C_{20}H_{14}O_2 + H)^+$, *m*/*z* 269 $(C_{20}H_{12}O + H)^+$, and *m*/*z* 253 $(C_{20}H_{12} + H)^+$ were selected to characterize benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), benzo[*a*]pyrene-7,8-dihydrodiol(±), 3-hydroxybenzo[*a*]pyrene, and benzo[*a*]pyrene. In operation of TOFMS-MS, a precursor ion spectrum was produced by scanning the first quadrupole, and a product ion spectrum was produced by holding the first quadrupole at constant *m*/*z* while scanning the tandem MS. With the optimized microflow LC-Q-TOFMS conditions as described in the experimental section, benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) was eluted at retention time 4–5 min and recorded through channel 1. Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±) was eluted at retention time 5–7 min and recorded through channel 2. Benzo[*a*]pyrene-7,8-dihydrodiol(±) was eluted at retention time 23–25 min and recorded through channel 3. 3-Hydroxybenzo[*a*]pyrene was eluted at retention time 24–26 min and recorded through channel 4. 6-Hydroxychrysene (ring $^{13}C_6$) used as an internal standard was eluted at retention time 30–32 min and recorded through channel 5. Benzo[*a*]pyrene was eluted at retention time 37–38 min and recorded through channel 6.

To further identify the analytes, the TOFMS-MS spectra were generated and analyzed. The TOFMS-MS measures a metastable transition or reaction from a metastable precursor ion to a product ion. Monitoring a metastable decomposition reaction provides a means by which to increase the selectivity of an assay for a given analyte. The increased selectivity derives from the additional dimension of tandem MS. A typical TOFMS-MS spectrum of benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) is presented in Fig. 1a and the collision energy was set at 10. During collision-induced dissociation, the ion under investigation was chosen as the precursor ion in the first MS of the quadrupole and directed into a collision cell, where it collided with a collision gas to acquire internal energy, which lead to its decomposition into product ions and detection by the second MS. The singly-charged benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) metabolite ion was observed at *m*/*z* 321.1298 $(C_{20}H_{16}O_4 + H)^+$. Three major fragments *m*/*z* 303.1604 that lost one H_2O from molecular ion, *m*/*z* 285.2782 that lost one H_2O from fragment *m*/*z* 303.1064, and *m*/*z* 257.2604 were observed under the experimental conditions. A typical TOFMS-MS spectrum of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±) is presented in Fig. 2a and the collision energy set at 10. The singly-charged metabolite ion was observed at *m*/*z* 303.1063 $(C_{20}H_{14}O_3 + H)^+$. Two major fragments *m*/*z* 285.0913 that lost one H_2O from molecular ion, and *m*/*z* 257.0923 were observed

under the experimental conditions. A typical TOFMS-MS spectrum of benzo[*a*]pyrene-7,8-dihydrodiol(±) is presented in Fig. 3a and the collision energy set at 12. The singly-charged metabolite ion was observed at *m*/*z* 287.1036 $(C_{20}H_{14}O_2 + H)^+$. Three major fragments *m*/*z* 269.0812 that lost one H_2O from molecular ion, *m*/*z* 258.0928, and *m*/*z* 241.0889 were observed under the experimental conditions. A typical TOFMS-MS spectrum of 3-hydroxybenzo[*a*]pyrene is presented in Fig. 4a. The singly-charged metabolite ion was observed at *m*/*z* 269.0980 $(C_{20}H_{12}O + H)^+$. Two major fragments *m*/*z* 251.1537 that lost one H_2O from molecular ion, and *m*/*z* 241.2089 were observed under the experimental conditions. A typical TOFMS-MS spectrum of benzo[*a*]pyrene is presented in Fig. 5a. The singly-charged molecular ion was observed at *m*/*z* 253.1040 $(C_{20}H_{12} + H)^+$. Two major fragments *m*/*z* 226.2933 and *m*/*z* 202.2284 were observed under the experimental condition. A typical TOFMS-MS spectrum of an internal standard molecular ion, 6-hydroxychrysene, was observed at *m*/*z* 251.2141 $(^{13}C_6C_{12}H_{12}O + H)^+$. Two major fragments *m*/*z* 231.2623 and *m*/*z* 209.2619 were observed.

Identification and quantification of urinary benzo[*a*]pyrene and its hydroxy-metabolites

A combination of microflow LC separation and a unique feature of tandem MS fragmentation pattern were used to determine the concentrations of benzo[*a*]pyrene and its metabolites of 3-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dihydrodiol(±), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), and benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) from mice urine. To achieve maximal detection sensitivity and selectivity, two criteria (1) retention time from microflow LC column, and (2) a characteristic fragmentation pattern of MS-MS spectrum, which matched the fragmentation of its reference standard, were set to quantify benzo[*a*]pyrene and its hydroxy-metabolites. Animal

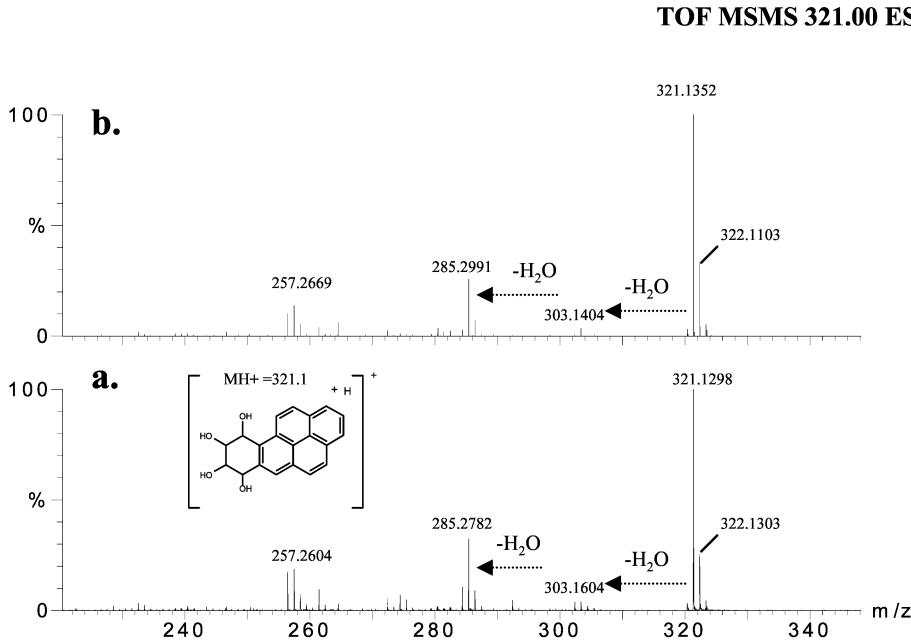

Fig. 1 A characteristic tandem MS fragmentation pattern of benzo[*a*]pyrene-7,8, 9,10-tetrahydrotetrol(±) metabolite detected from (a) a reference standard solution, and (b) urine of asphalt fume exposed mice.

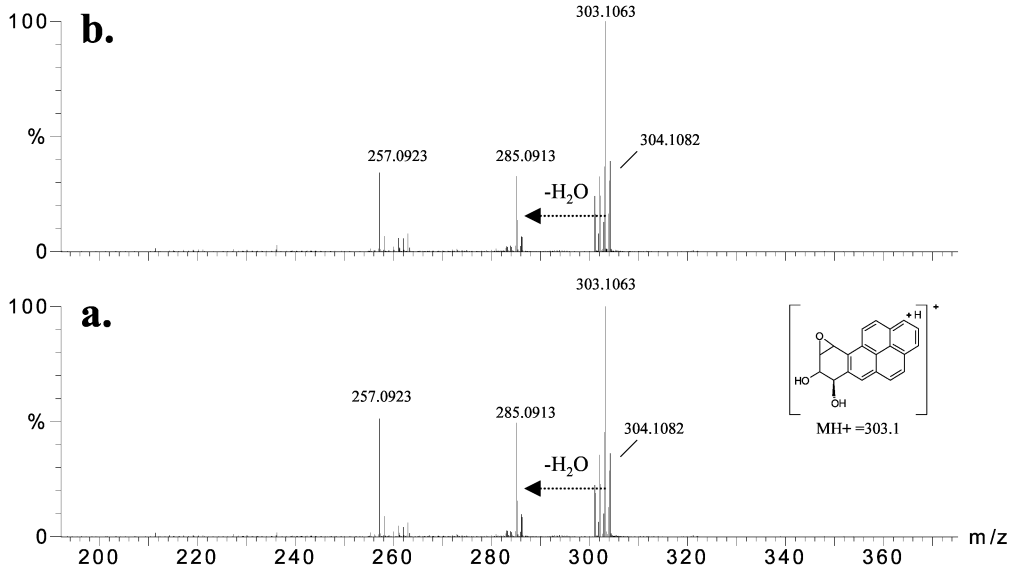

Fig. 2 A characteristic tandem MS fragmentation pattern of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±) metabolite detected from (a) a reference standard solution, and (b) urine of asphalt fume exposed mice.

exposure trials were conducted twice (16 mice for each period) with the same exposure conditions (exposure time and temperature of fume generation). To evaluate the recovery of the target analytes, a stable isotope 6-hydroxychrysene (ring $^{13}C_6C_{12}H_{12}O$) was used as an internal standard. The relative recovery of the internal standard can account for losses of the analytes during sample preparation and detection processes. The recovery of spiked reference standard metabolites was 72%, and slightly lower recovery was observed for benzo[*a*]-pyrene-7,8-dihydrodiol(±). Following the optimization procedure, quantitative results were based on calculations by a reference standard with 4 point measurements. The results of experimentally determined benzo[*a*]pyrene and its hydroxy-metabolites were summarized in Table 1. Benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) was determined 45.23 ng 100 mL^{-1} urine from exposed mice at retention time 4–5 min and recorded through channel 1.

A typical TOFMS-MS spectrum from exposed urine is presented in Fig. 1b. The singly-charged benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) metabolite ion was observed at *m*/*z* 321.1352 $(C_{20}H_{16}O_4 + H)^+$. Three major fragments *m*/*z* 303.1404 that lost one H_2O from molecular ion, *m*/*z* 285.2991 that lost one H_2O from ion *m*/*z* 303.1404, and *m*/*z* 257.2669 were observed that were identical to those from benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) reference standard solution (Fig. 1a). Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-expoxide(±) was determined at 54.92 ng 100 ml^{-1} urine from exposed mice at retention time 5–7 min and recorded through channel 2. A typical TOFMS-MS spectrum from exposed urine is presented in Fig. 2b. The singly-charged benzo[*a*]pyrene-7,8-dihydrodiol-9,10-expoxide(±) metabolite ion was observed at *m*/*z* 303.1063 $(C_{20}H_{14}O_3 + H)^+$. Two major fragments *m*/*z* 285.0913 that lost one H_2O from molecular ion, and *m*/*z* 257.0923 were observed that were identical to those from the reference standard solution (Fig. 2a). Benzo[*a*]pyrene-7,8-dihydrodiol(±) was determined at 11.56 ng 100 mL^{-1} urine from exposed mice at retention time 23–25 min and recorded through channel 3. A typical TOFMS-MS spectrum from exposed urine is presented in Fig. 3b. The singly-charged benzo[*a*]pyrene-7,8-dihydrodiol(±) metabolite ion was observed at *m*/*z* 287.1166 $(C_{20}H_{14}O_2 + H)^+$. Three

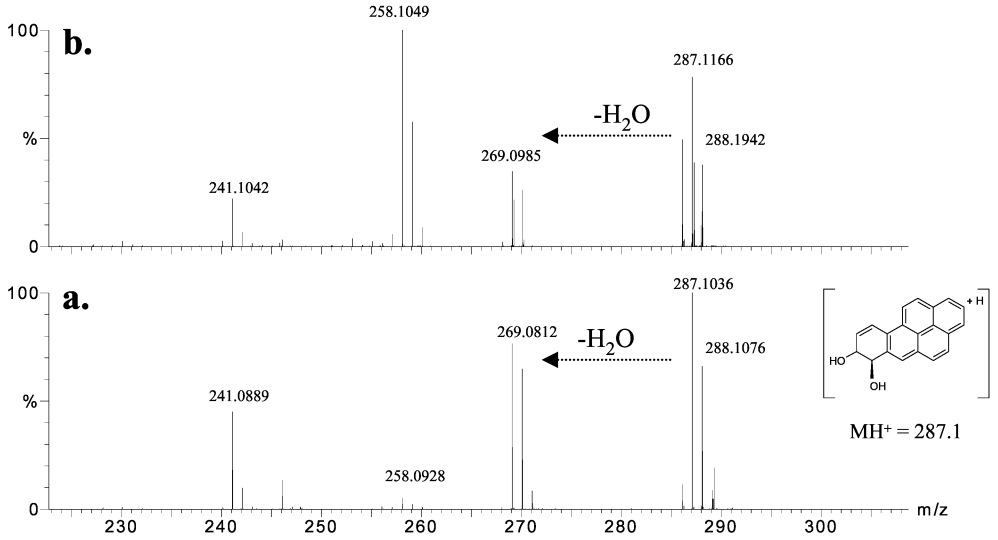

Fig. 3 A characteristic tandem MS fragmentation pattern of benzo[*a*]pyrene-7,8-dihydrodiol(±) metabolite detected from (a) a reference standard solution, and (b) urine of asphalt fume exposed mice.

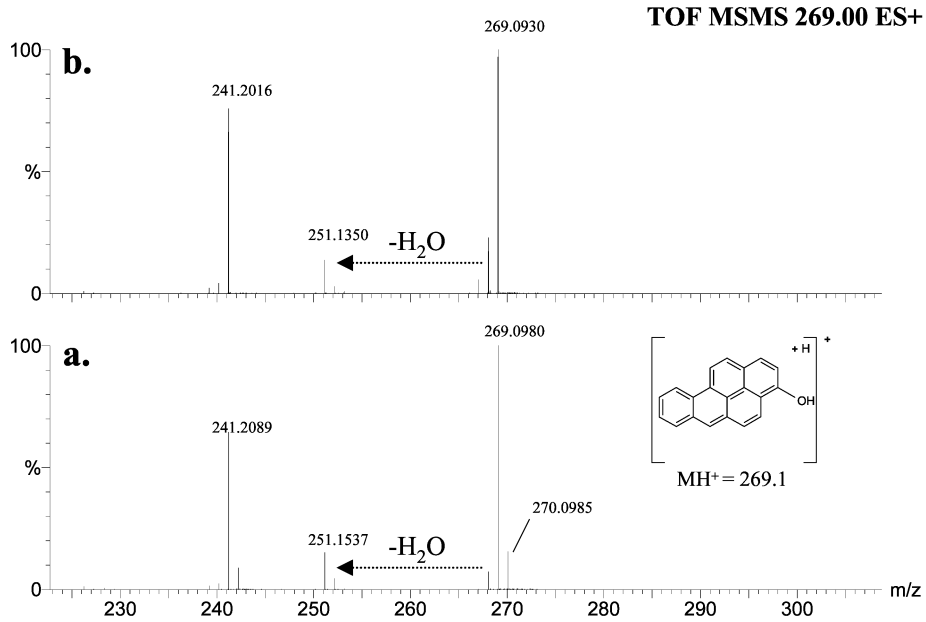

Fig. 4 A characteristic tandem MS fragmentation pattern of 3-hydroxybenzo[*a*]pyrene metabolite detected from (a) a reference standard solution, and (b) urine of asphalt fumes exposed mice.

major fragments *m*/*z* 269.0985 that lost one H_2O from molecular ion, *m*/*z* 258.1049, and *m*/*z* 241.1042 were observed that were identical to those from the reference standard solution (Fig. 3a). In view of the intensities of these fragments in Fig. 3, it was noticed that the relative intensities of parent ion to fragments *m*/*z* 269, and 258 in the MS-MS spectra from exposed urine were different from those of the standard solution. The variations of relative intensity of fragments may be explained by the isomers of benzo[*a*]pyrene-7,8-dihydrodiol(±) or some interferences from urine sample. Nevertheless, the observed intensity variations do not change the fundamental fragmentation pattern. The benzo[*a*]pyrene-7,8-dihydrodiol(±) can still be identified based on its specific fragmentation pattern. In this experiment, 3-hydroxybenzo[*a*]pyrene was determined 31.36 ng 100 mL^{-1} urine from exposed mice at retention time 24–26 min and recorded through channel 4.

A typical TOFMS-MS spectrum from exposed urine is presented in Fig. 4b. The singly-charged 3-hydroxybenzo[*a*]-pyrene metabolite ion was observed at *m*/*z* 269.0930 ($C_{20}H_{12}O$ + $H)^+$. Two major fragments *m*/*z* 251.1350 that lost one H_2O from molecular ion, and *m*/*z* 241.2016 were observed that were identical to those from the reference standard solution (Fig. 4a). Benzo[*a*]pyrene was determined at 3.18 ng 100 mL^{-1} urine from exposed mice at retention time 36–38 min and recorded through channel 6. A typical TOFMS-MS spectrum is presented in Fig. 5b. The singly-charged benzo[*a*]pyrene molecular ion was observed at *m*/*z* 253.1096 ($C_{20}H_{12}$ + $H)^+$. Two major fragments *m*/*z* 226.2990 and *m*/*z* 202.2340 were observed that were identical to those from benzo[*a*]pyrene reference standard solution (Fig. 5a). 6-Hydroxychrysene acted as an internal standard and it was detected at retention time 30–32 min and recorded through channel 5. Molecular ion 6-hydroxychrysene (*m*/*z* 251.2143), and two major fragments (*m*/*z* 231.2603 and *m*/*z* 209.2609) were observed from a typical TOFMS-MS spectrum. The relative standard deviation for asphalt fume exposed urine over 10 days ranged 19–28%.

The control urine samples were analyzed under the same experimental conditions. The concentrations of benzo[*a*]-pyrene, benzo[*a*]pyrene-7,8-dihydrodiol(±), and 3-hydroxy-benzo[*a*]pyrene were under the detection limits, except that the

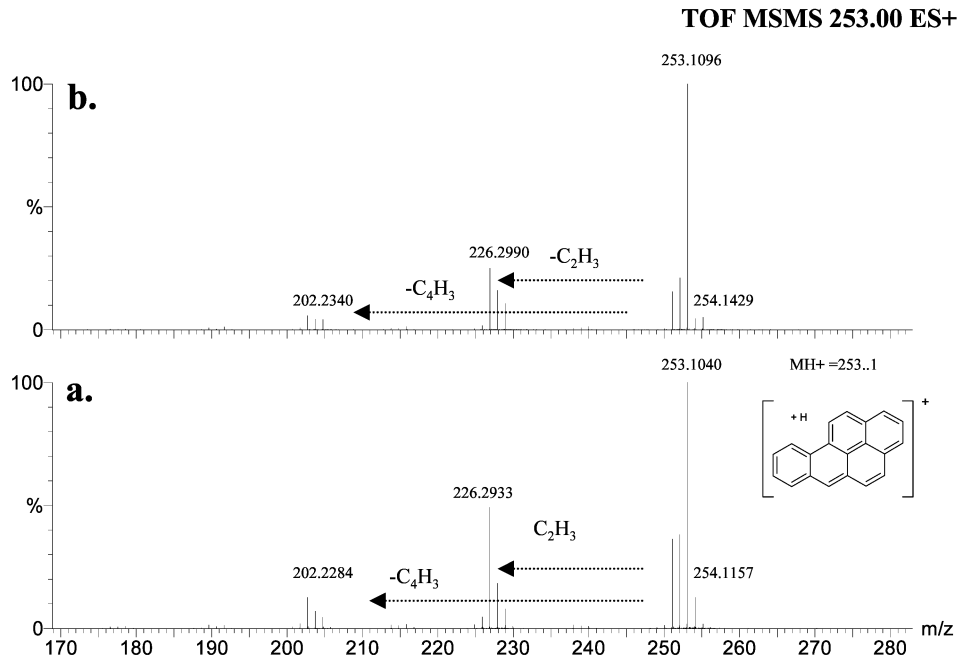

Fig. 5 A characteristic tandem MS fragmentation pattern of benzo[*a*]pyrene detected from (a) a reference standard solution, and (b) urine of asphalt fume exposed mice.

Table 1 Determined concentrations of benzo[*a*]pyrene and its hydroxy-metabolites from urine of asphalt fume exposed mice

Benzo[*a*]pyrene and its hydroxy-metabolite	Major *m*/*z* found	Exposed urine/ ng 100 mL^{-1} ± RSD[a]	Control urine/ ng 100 mL^{-1} ± RSD
Benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±)	321.1352 303.1404 285.2991 257.2669	45.23 ± 0.19	BDL[b]
Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±)	303.1063 285.0913 257.0923	54.92 ± 0.21	0.21 ± 0.29
Benzo[*a*]pyrene-7,8-dihydrodiol(±)	287.1166 269.0985 258.1049 241.1042	11.56 ± 0.19	BDL
3-Hydroxybenzo[*a*]pyrene	269.0930 251.1350 241.2016	31.36 ± 0.25	BDL
Benzo[*a*]pyrene	253.1096 226.2990 202.2340	3.18 ± 0.28	BDL

[a] RSD = relative standard deviation. [b] BDL = below detection limit.

concentration of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±) metabolite was determined at 0.21 ng 100 mL^{-1} urine from control group mice. In summary, the results indicated that the urinary benzo[*a*]pyrene, and its hydroxy-metabolites were significantly increased ($p < 0.001$) from asphalt fume exposed mice.

Several other techniques such as HPLC-UV and HPLC-fluorescence detections have also been used to analyze the benzo[*a*]pyrene and its metabolites. These techniques are convenient to use and cost-effective. The major limitation of those methods is that they cannot provide specific information on the chemical identity and the toxic progenitor that is essential to obtain a more comprehensive understanding of how the hazards attribute to toxicity and cause adverse health effects. The positive ionization microflow LC-Q-TOFMS technique does not have such a limitation. This technique makes it possible to move at least one step further towards understanding the mechanisms of the adverse health effects associated with the asphalt fume exposure. Furthermore, the limit of detection in this study is in the range of 40–60 pg based on a three-fold signal-to-noise ratio, which is much more sensitive than those of the regular flow UV and fluorescence detections.

Determination of asphalt fume in animal exposure chamber

Asphalt fume in animal exposure chamber was determined by positive electron ionization and selected ion monitoring of GC-MS respectively. The relative recovery of the internal standard can account for losses of the analytes during sample preparation and detection. The cumulative exposure to asphalt fume over 10 days was summarized in Table 2. The second column of Table 2 reported asphalt fume particulates collected on filters and ranged from 62.69 to 70.64 mg m^{-3}. The third column presented the quantities of asphalt fume that were trapped on XAD-2 tubes and ranged from 104.56 to 119.65 mg m^{-3}. The total asphalt fume concentrations were summed in the fourth column and ranged from 175.20 to 182.34 mg m^{-3}. The relative standard deviation of total fume over exposure 10 days ranged from 7.1–21.9%.

Conclusions

A bioanalytical method was proposed and evaluated to characterize PAH metabolites. It is based on a microflow LC coupled to a Q-TOFMS. Compared with several other technologies such as HPLC-UV and HPLC-fluorescence, the new method is more sensitive and selective, and it can also provide additional useful information on the structures of the metabolites. The use of collision-induced dissociation leading to obtaining a characteristic fragmentation pattern represents a new approach to characterize and elucidate molecular structural features of the PAH metabolites. This method can thus be used to help understand the mechanisms of the toxicity of the asphalt fume. This method may also help the integration of mass spectrometry into work-related research studies and the measurement for risk assessment of asphalt fume exposure.

Benzo[*a*]pyrene and its metabolites of 3-hydroxybenzo[*a*]-pyrene, benzo[*a*]pyrene-7,8-dihydrodiol(±), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide(±), and benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol(±) were found significantly higher ($p < 0.001$) from asphalt fume exposed urine than those from control animals. The results indicated that the asphalt fume exposure might cause potential adverse health effects. The information obtained from this study may help improve our understanding of the molecular mechanisms of adverse health effects and assist the risk assessment of asphalt fume exposure. Furthermore, the fragmentation patterns established in this study may also be used to identify and quantify PAH metabolites in other biological systems.

Table 2 Determined asphalt fume concentrations in mice exposure chamber

Asphalt exposure day[a]	Asphalt fume by filter/ mg m^{-3} ± RSD%[bc]	Asphalt fume by XAD-2/ mg m^{-3} ± RSD%	Asphalt fume total/ mg m^{-3} ± RSD%
1–5	70.64 ± 14.13	104.56 ± 28.85	175.20 ± 21.90
6–10	62.69 ± 12.54	119.65 ± 9.89	182.34 ± 7.10

[a] Ten exposure days, 4h day^{-1}. [b] RSD = relative standard deviation. [c] Quantitative results of asphalt fume based on calibration with a PAHs mixture.

Acknowledgement

We gratefully thank Al Munson, Paul Siegel, Nancy Bollinger, Jean Meade, Beverly Carter, and Judy Mull for their project support and coordination. We also thank Aaron Timperman and Vo Evanly for providing critical review of the manuscript.

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