# Extraction and Purification of Depurinated Benzo[*a*]pyrene-Adducted DNA Bases from Human Urine by Immunoaffinity Chromatography Coupled with HPLC and Analysis by LC/Quadrupole Ion-Trap MS

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In this paper, we describe implementation and testing of an immunoaffinity (IA) column for rapid and selective extraction of 7-(benzo[*a*]pyren-6-yl)adenine (BP-6-N7Ade) and 7-(benzo[*a*]-pyren-6-yl)guanine (BP-6-N7Gua) from urine, where BP is benzo[*a*]pyrene. The BP radical cation is a carcinogenic metabolite that reacts with double-stranded DNA, producing depurinated BP-adducted DNA bases excreted in urine. The expected modified nucleobases are BP-6-N7Gua, BP-6-N7Ade, and 8-(benzo[*a*]pyren-6-yl)guanine (BP-6-C8Gua), and they may serve as important biomarkers for DNA damage by PAHs. IA extracts of urine from a cigarette smoker and a nonsmoker contained less than 5% of contaminants present in Sep-Pak extracts and, unlike the latter, were suitable for analytical HPLC. IA extraction achieved 75−95% recovery of BP-6-N7Gua (10 fmol/mL) and BP-6-N7Ade (1 fmol/mL) added to urine samples. Tandem mass spectrometry of IA/HPLC fractions of urine from two coal smoke-exposed women at high risk for lung cancer demonstrated the presence of 20 and 50 fmol BP-6-N7Gua per mL of urine. Unexposed controls were negative. With proposed modifications, the IA-based protocol can achieve a detection limit of 0.1 fmol/mL urine, which is sufficient for routine quantification of BP-adducted bases in urine of cigarette smokers. This procedure may allow screening of persons at risk for lung cancer associated with exposure to PAH in cigarette and other forms of smoke.

## Introduction

Lung cancer, one of the most aggressive neoplasms, is the leading cause of cancer deaths among men and women living in the United States. In 1998, cancers of the lung caused an estimated 160 100 deaths including 93 100 deaths among men and 67 000 deaths among women (*1*). Despite all efforts at early diagnosis, the overall 5 year survival rate is ca. 9% (*2*). A causal link between cigarette smoking and lung cancer is well-established (*3*). The Surgeon General's report of 1989 (*4*) estimated that cigarette smoking accounted for 90% of lung cancer in males and 79% in females. The attributed risk for females is increasing with increasing prevalence of smoking in this group. A number of case control and cohort studies in the 1970s and 1980s reported significant reduction in lung cancer risk for long-term smokers of filter cigarettes. Recent studies have found, however, no significant difference in lung cancer risk for smokers of low yield cigarettes in comparison to smokers of high yield cigarettes (*3*).

Clearly, there is a need for more effective approaches to reducing lung cancer incidence. Identification of individual smokers at high risk is a potentially effective strategy targeting risk-taking behavior. This requires development of effective assessment protocols based on our knowledge of the chemistry of cigarette smoke and mechanisms of carcinogenesis induced by smoke constituents.

Tobacco smoke contains ca. 4000 compounds including ca. 40 known carcinogens (*5*). BP,[1] a very potent carcinogen in the PAH family, is present in the particulate fraction of cigarette smoke. It is widely accepted as an index carcinogen for determining risk of PAH-associated cancers. BP is invariably present in cigarette smoke, which delivers 20−40 ng per cigarette, and ranks as one of the most potent carcinogens in that smoke. Smoke particulates colocalize with the large majority of smoking-induced lung tumors, implicating BP and other carcinogenic PAH as principal causes of smoking-induced lung cancer. In addition, mutations in the p53 gene, which occur in ca. 60% of lung cancers in cigarette smokers, reflect hotspots of BP adduction (*6*).

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[1] Abbreviations: ATS, 3-aminotriethoxysilane; AUC, area under curve; BP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene-7,8-diol-9,10-epoxide; BP-6-N7Gua, 7-(benzo[*a*]pyren-6-yl)guanine; BP-6-N7Ade, 7-(benzo[*a*]pyren-6-yl)adenine; BP-6-C8Gua, 8-(benzo[*a*]pyren-6-yl)-guanine; DMEM, Dulbecco's minimal essential medium; ECS, extracapillary space of the CellMax cartridge; FBS, fetal bovine serum; IA, immunoaffinity; IAC, immunoaffinity chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; Mab, monoclonal antibody; MS/MS, tandem mass spectrometry; PAH, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; TNBS, trinitrobenzene sulfonic acid.

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BP is metabolized by cytochrome P450s to BPDE, a carcinogenic electrophile that forms stable adducts with DNA, RNA, and protein (*7*, *8*). BPDE adducts to DNA, hemoglobin, and serum albumin have been evaluated as biomarkers of internal dose of metabolically activated BP and are viewed as indicators of cancer risk (*9*). Other carcinogenic PAHs are similarly activated to electrophilic diol epoxides. Stable PAH or other aromatic adducts to DNA may serve as biomarkers of individual risk of PAH-associated cancers (*10*).

PAHs also are metabolized by cytochrome P450s and peroxidases to radical cations that bind covalently to C-8 or N-7 of guanine or to N-3 or N-7 of adenine. Formation of these adducts, requiring double-stranded DNA, destabilizes the *N*-glycosyl bond to the deoxyribose phosphate backbone of DNA and leads to the formation of apurinic sites (*8*). When mouse skin was treated with BP, 66% of all BP−DNA adducts were depurinating adducts formed by the BP radical cation. These adducts include BP-6-N7Gua (10%), BP-6-C8Gua (34%), and BP-6-N7Ade (22%). Carcinogenecity studies of BP and BP metabolites have established a quantitative correlation between carcinogenic potency and formation of depurinating DNA adducts (*8*). In addition, mutagenicity studies of BP and other carcinogenic PAH support depurination as an essential mechanism producing mutations in the H-*ras* oncogene (*11*).

Recently, we identified BP-6-N7Gua and BP-6-N7Ade in the urine of cigarette smokers and individuals exposed to household coal smoke (*10*). In adduct positive individuals, depurinated BP-adducted DNA bases displayed a strong quantitative correlation with PAH exposure. Urinary concentrations were estimated to be 60−340 fmol/mg of creatinine for coal smoke-exposed women (BP intake of ca. 23 000 ng/day) and 0.1−0.6 fmol/mg creatinine for cigarette smokers (BP intake of ca. 800 ng/day). Adducted bases were not detected in the urine of control subjects. The results justify larger scale studies of the distribution of depurinated BP−DNA bases in PAH-exposed and nonexposed individuals. This will require a rapid and efficient protocol for detection and quantification of these bases in urine samples. To this end, we have developed an IA protocol for efficient extraction of BP-6-N7Gua and BP-6-N7Ade from urine samples.

In this paper, we present the results of our work on (i) validation of an IAC/HPLC protocol for highly selective extraction of BP-6-N7Gua and BP-6-N7Ade from human urine and (ii) application of this protocol to detection of BP-6-N7Gua and BP-6-N7Ade in urine of cigarette smokers and individuals exposed to household coal smoke.

## Experimental Procedures

**Chemicals.** The BP-adducted bases BP-6-N7Ade and BP-6-N7Gua were synthesized by iodine oxidation of BP in the presence of 2′-deoxyguanosine, 2′-deoxyadenosine, or Ade (*12*). These served as standards for protocol development and for detection and quantification of depurinated BP-adducted DNA bases in human urine.

**Collection of Urine Samples.** Study subjects were asked to provide urine first passed in the morning. Each study subject completed a consent form and a questionnaire that provided the following information: age, sex, history of tobacco use, health status, and dietary and occupational exposure to PAH. Our studies were carried out according to recommendations of the World Medical Association Declaration of Helsinki. They were approved by (i) the Institutional Review Board for the Protection of Human Subjects, University of Nebraska Medical Center, and (ii) the U.S. Environmental Protection Agency for international research projects.

Urine samples were collected from two Chinese women (ages 44 and 49 years) exposed to coal smoke and two "nonexposed women" (ages 36 and 54 years). The exposed subjects, who maintained households in Xuan Wei, China, were exposed to unvented smoke produced by combustion of smoky coal, which served as fuel for both household heating and cooking. Twenty-four hour mean concentrations of household BP were 303−1970 ng/m$^3$ of air. In addition, for as many as 7 h per day, these women were near the coal fire where the average concentration of BP was 14 650 ng/m$^3$ of air (*13*). Nonexposed controls, comprising two Chinese-American women, reported only ambient exposure to air-born PAH. Neither the exposed subjects nor the nonexposed controls used tobacco products, consumed (2 weeks before urine collection) foods rich in PAH, or experienced occupational exposure to PAH.

Urine samples were collected from one smoker (male, age 46 years) and one nonsmoker (male, age 57 years) at the Pulmonary and Critical Care Medicine Section of the Department of Internal Medicine, University of Nebraska Medical Center. The smoker consumed an average of 40 filtered cigarettes per day. The nonsmoker indicated only ambient exposure to PAH and did not have a recent (20 years or more) history of using tobacco products. Neither subject experienced occupational exposure to PAH nor consumed (2 weeks before urine collection) foods containing high concentrations of PAH.

The creatinine concentration of each urine sample was determined by a colorimetric procedure (Stanbio Creatinine Procedure No. 0400, Sigma-Aldrich, St. Louis, MO), upon receipt into the laboratory. All urine samples were stored at −80 °C.

**Production of an IA Matrix Consisting of Mab CB53 Coupled to Glass Beads.** Mab CB53 (700−800 mg) was produced with the CellMax artificial capillary system (Spectrum Laboratories, Rancho Dominguez, CA). This antibody has a high specificity and affinity for both BP-6-N7Gua and BP-6-N7Ade (*14*). Briefly, hybridoma CB53 was seeded to the ECS of the CellMax cartridge. High-glucose DMEM with 10% FBS was continuously circulated through the porous capillaries of the cartridge. By the end of the first 2 weeks of culture, the cells had adapted to 2.5% FBS and the system had achieved maximal antibody production (ca. 120−150 mg/week). Antibody present in the ECS was purified (95% purity) by ammonium sulfate precipitation, dissolved in PBS (pH 7.0) to a concentration of 5−10 mg/mL, dialyzed against PBS, and stored at −80 °C. Purity of the antibody preparations was determined by densitometry of samples fractionated by SDS−PAGE and then stained with Coomassie Blue (Bio-Rad Laboratories, Hercules, CA).

Acid-washed glass beads (12 g) with a mean diameter of 106 $\mu$m (Sigma-Aldrich, Inc.) were introduced into a Pyrex tube and heated overnight at 125 °C. Five milliliters of a 4% (v/v) solution of ATS (Sigma-Aldrich, Inc.) in HPLC grade acetone (Burdick and Jackson, Muskegon, MI) was added to the beads at room temperature. The tube was sealed with a foil-lined cap and heated at 40 °C for 48 h. The ATS-treated beads were washed in sequence with 120 mL of acetone, 200 mL of $H_2O$, and 120 mL of acetone and then dried overnight at 50 °C.

A sample of processed beads was evaluated for successful derivatization with ATS. Processed beads (0.2 g) and untreated beads (0.2 g) were introduced into 1.5 mL polypropylene Eppendorf tubes and treated with 150 $\mu$L of 0.003% TNBS (Sigma-Aldrich, Inc.) in 0.1 M sodium bicarbonate buffer at pH 8.5. The tubes were sealed and then rotated end-over-end for 2 h at 37 °C. The reaction mixtures were acidified with 25 $\mu$L of 1 M HCl and observed for color. Processed beads exhibited a bright yellow color produced by coupling of TNBS with the primary amino group of ATS. In contrast, untreated beads were colorless.

Glutaraldehyde, which would serve to link antibody to ATS-derivatized beads, was coupled to the balance (ca. 12 g) of the

ATS beads. The latter were combined with a mixture of 7.5 mL of 25% glutaraldehyde (EM grade; Sigma-Aldrich, Inc.) and 7.5 mL of 0.5 M borate buffer (pH 9.0), in a screw cap Pyrex tube. Sodium cyanoborohydride ($NaCNBH_3$) was added at 0.2 mg/mL, and then, the tube was sealed with a foil-lined cap and rotated end-over-end for 24 h at 37 °C. At the end of this period, the supernatant was removed and the beads were washed with 2 L of $H_2O$.

A sample of washed beads was evaluated for the presence of amino-coupled glutaraldehyde. The beads (0.2 g) were added to each of two 2.0 mL Eppendorf tubes and washed with 10 mL of borate buffer. One sample was treated with 0.2 mL of borate buffer, and the other sample was treated with a 0.2 mL of a solution containing L-Lys (0.5 M) and $NaCNBH_3$ (0.2 mg/mL) in borate buffer. The tubes were sealed and rotated end-over-end for 24 h at 37 °C. The samples were evaluated for the presence of primary amino groups as described above. The L-Lys-treated sample exhibited a bright yellow color, in contrast to the borate buffer control, which was colorless.

Glutaraldehyde-substituted ATS beads were washed by filtration on a scintered glass filter with 100 mL of 0.1 M sodium bicarbonate buffer. The beads were brought to near dryness and then transferred to a fresh Pyrex tube. Mab CB53 (30 mg) (*14*) dissolved in 10 mL of bicarbonate buffer was introduced into the tube. The latter was sealed and rotated end-over-end for 24 h at 37 °C. The antibody-coated beads were washed with 180 mL of bicarbonate buffer and suspended in 10 mL of this buffer, and then, unreacted aldehyde groups were blocked by addition of 60 $\mu$L of ethanolamine. Again, the tubes were rotated end-over-end for 24 h at 37 °C. Finally, the beads were washed with 180 mL of 0.05 M Tris buffer (pH 7.5) and suspended in 10 mL of the same buffer.

Antibody-coated beads (prior to ethanolamine treatment) were evaluated for the presence of coupled protein with BCA reagent (Pierce, Rockford, IL). Coated beads (0.2 g) and untreated beads (0.2 g) were introduced into 1.5 mL polypropylene Eppendorf tubes, followed by addition of 500 $\mu$L of BCA reagent. The tubes were rotated end-over-end for 40 min at 37 °C. The supernatant above the coated beads exhibited a purple color, in contrast to the supernatant above the untreated beads, which was colorless.

**IAC Extraction of BP-Adducted DNA Bases in Human Urine.** A suspension containing 3 g of antibody-coated glass beads was introduced into individual 1.0 cm × 10 cm Econo-Columns (BioRad, Hercules, CA), and the beads were allowed to settle. This yielded a packed volume of ca. 3 mL per column. The IA columns were washed with 100 mL of Tris buffer and then capped and kept at room temperature. If the columns were not to be used within 48 h, they were stored for up to 8 weeks at 4 °C in Tris buffer containing 0.02% sodium azide.

Urine samples, stored at −80 °C, were thawed in a 37 °C water bath, adjusted to pH 7.0 with 3 M HCl, and stored at 4 °C. The next day, the samples were warmed in a 37 °C water bath for 30−45 min and then clarified at room temperature by centrifugation at 1800 rpm for 10 min. One urine sample from a smoker and a nonsmoker provided 2 vol of 80 mL each. One volume was treated by addition of 0.1 pmol of BP-6-N7Ade and 1.0 pmol of BP-6-N7Gua. The remaining volume was left untreated. One 15 mL sample from each of two coal smoke-exposed women and one 20 mL sample from each of two Chinese-American controls were left untreated. Each urine sample was passed through its own IA column, at a flow rate of 0.5 mL/min. Subsequently, the column was washed with 200 mL of Tris buffer, at a flow rate of 2.0 mL/min. BP-adducted DNA bases that may have been present in the urine sample were eluted from the column with 100% acetonitrile. Ten milliliters of $CH_3CN$ was applied to the column, and the $CH_3CN$ was allowed to percolate (ca. 2 mL) into the matrix, displacing the Tris buffer. The flow was stopped for 30 min. At the end of this period, the eluate was collected at a rate of 2.0 mL/min. This procedure was repeated two more times, yielding a total volume of 30 mL. The eluate was extracted with chloroform, the extract was rotary-evaporated into 100 $\mu$L of dimethyl sulfoxide ($Me_2SO$), and the latter was reduced to 10 $\mu$L under high vacuum.

**Fractionation and Analysis of IAC Extracts of Urine by HPLC.** Urine samples, prepared by IAC extraction, were fractionated by reverse phase HPLC with a Waters 625 LC System (Waters Inc., Milford, MA). Each 10 $\mu$L sample was applied to a 2.0 mm × 250 mm YMC-ODS-AQ column (Waters Corp., Milford, MA) fitted with a 2.0 mm × 20 mm YMC-ODS-AQ direct connect guard cartridge (Waters Corp.) and equilibrated with $CH_3CN/H_2O$ (35:65). The column was eluted at 0.2 mL/min with the following program: (i) 0−5 min, $CH_3CN/H_2O$ (35:65); (ii) 5−65 min, a linear gradient to $CH_3CN/H_2O$ (55:45); (iii) 65−75 min, a linear gradient to 100% $CH_3CN$; and (iv) 75−90 min, 100% $CH_3CN$. The column eluate was monitored at 427 nm with a Waters 474 Scanning Fluorescence Detector (Waters Inc.) set to excite at 365 nm. The column eluate was collected from 42 to 52 min, a region that encompassed the retention times of BP-6-N7Gua and BP-6-N7Ade. To ensure the absence of carry-over from one fractionation to the next, we injected the HPLC column with $Me_2SO$, alone, after each sample fractionation, and collected the same fraction. All fractions were extracted with $CHCl_3$, concentrated to 10 $\mu$L in $Me_2SO$, and then analyzed by a second HPLC program described in the following paragraph.

Samples (10 $\mu$L) were applied to a 2.0 mm × 250 mm YMC-ODS-AQ column (Waters Corp.) fitted with a 2.0 mm × 20 mm YMC-ODS-AQ direct-connect guard cartridge (Waters Corp.). The column was equilibrated with 40% solvent A [1/3EtOH + 2/3$CH_3CN$ + 0.2% (v/v) acetic acid] and 60% acidified $H_2O$ (containing 0.2% acetic acid). The column was eluted at 0.2 mL/min with the following program: (i) 0−10 min, solvent A/acidified $H_2O$ (40:60); (ii) 10−120 min, a linear gradient to 100% solvent A; and (iii) 120−165 min, 100% solvent A. The column eluate was collected from 32 to 42 min, a region that encompassed the retention times of BP-6-N7Gua and BP-6-N7Ade. The absence of carry-over from one fractionation to the next was confirmed by the absence of fluorescence emission in eluate from the HPLC column injected with $Me_2SO$ after each sample fractionation. All fractions were extracted with $CHCl_3$, concentrated to 20 $\mu$L in $Me_2SO$, and then shipped to the mass spectrometry laboratory for analysis.

**Identification and Quantification of Urinary BP-6-N7Gua and BP-6-N7Ade by LC/Quadrupole Ion-Trap Mass Spectrometry.** HPLC fractions of urine were analyzed for BP-6-N7Gua and BP-6-N7Ade twice at different times using slightly different procedures. For both analyses, solvent changes over time were linear. Samples in $Me_2SO$ were diluted 50/50 (v/v) with starting buffer. Samples (10 $\mu$L) were injected via a Rheodyne 7125 injector onto a 0.3 mm × 150 mm Zorbax $C_{18}$ column (MicroTech, Sunnyvale, CA) fitted with a 0.3 mm × 10 mm guard column of the same packing material. For one set of results, the column was eluted as follows. Solvent A ($H_2O$) was kept constant at 75% for the first 5 min, was decreased to 30% in the next 5 min, and then decreased to 10% in the next 20 min. Solvent B (50/50 2-propanol/methanol) was kept constant at 15% for the first 5 min, was increased to 60% in the next 5 min, and then increased to 80% in the next 20 min. Solvent C (10% $CH_3COOH$, 100 mM $NH_4OCOCH_3$, and $H_2O$) was kept constant at 10% for the entire run. The final solvent composition was kept constant for the next 5 min to elute all sample components. The column was eluted at a flow rate of 5.0 $\mu$L/min. Solvent flow from the HPLC pump (Waters 600MS with silk pulse dampening enabled) was split with an LC Packings Accurate Splitter fitted with a 0.3 mm column calibrator (LC Packings, San Francisco, CA).

For the second set of results, the column was eluted as follows. Solvent A ($H_2O$) was kept constant at 84% for the first 5 min, was decreased to 39% in the next 5 min, and then decreased to 19% in the next 20 min. Solvent B (50/50 2-propanol/methanol) was kept constant at 15% for the first 5 min, was increased to 60% in the next 5 min, and then increased to

80% in the next 20 min. Solvent C (10% $CH_3COOH$, 100 mM $NH_4OCOCH_3$, and $H_2O$) was kept constant at 1% for the entire run. The final solvent composition was kept constant for the next 5 min to elute all sample components. The column was eluted at a flow rate of 5.0 $\mu$L/min. Solvent flow from the HPLC pump (Waters 600MS with silk pulse dampening enabled) was split with an LC Packings Accurate Splitter fitted with a 0.3 mm column calibrator (LC Packings).

All of the column eluent was introduced into a Finnigan LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA), by electrospray ionization. Eluent from the HPLC column was sprayed into a heated capillary (200 °C), the spray voltage was 4.5 kV, and the nitrogen sheath gas flow was 60 mL/min. The mass spectrometer was set with one acquisition segment that included three scans. The first was a full MS scan of the range *m/z* 200−450. The second was a scan (MS/MS mode) of product ions produced from protonated BP-6-N7Gua ($[M + H]^+$; *m/z* 402.2) isolated in a window 1.5 *m/z* wide. The third was a scan (MS/MS mode) of product ions produced from protonated BP-6-N7Ade ($[M + H]^+$; *m/z* 386.2) isolated in a window 1.5 *m/z* wide. The resonant excitation energy was set at 40% of maximum, and the MS/MS scan ranges were set to *m/z* 210−410 for BP-6-N7Gua and *m/z* 200−390 for BP-6-N7Ade. Spectra were acquired in the "profile" mode, and each spectrum was the average of two 1000 ms scans. BP-6-N7Gua was considered present in a sample if its principal fragment ions (those of *m/z* 252, 360, and 385) were detected. BP-6-N7Ade was considered present if its principal fragment ions (those of *m/z* 369, 342, and 252) were detected. Signals elsewhere in the product ion mass spectra represented ions of coeluting interferences.

Concentrations of BP-6-N7Gua were estimated with a calibration plot of peak area of the *m/z* 252 ion vs quantity of standard injected. Concentrations of BP-6-N7Ade were estimated with a calibration plot of peak area of the *m/z* 369 ion vs quantity of standard injected. The plots were linear over a range of 5−25 fmol.

Abundance ratios were variable because the fragments were present so close to their detection limits, and consequently, ion-counting statistics were low. Signals elsewhere in the tandem mass spectra represent fragment ions of coeluates and not electronic noise.

## Results

**IAC Protocol for Selective Extraction of BP-6-N7Gua and BP-6-N7Ade from Human Urine: Evaluation by HPLC.** Our previous study (*10*) indicated the need for a simple and effective extraction with far greater selectivity for BP-6-N7Gua and BP-6-N7Ade than that of SepPak extraction. To meet this need, we designed this study to examine the effectiveness of IAC extraction of BP-6-N7Gua and BP-6-N7Ade standards added to human urine samples, at concentrations consistent with those produced by environmental exposures to BP (*10*). SepPak fractions of urine samples (ca. 80−100 mL) sufficient for detection of depurinated BP-adducted bases in cigarette smokers or coal smoke-exposed individuals overload analytical HPLC columns, yielding extreme, off-scale fluorescence signals over the time course of analysis (data not shown).

A mixture of 1.0 pmol of BP-6-N7Gua and 0.1 pmol of BP-6-N7Ade was added to 80 mL of urine collected from a cigarette smoker and a nonsmoker. BP-adducted bases were not added to corresponding control samples from the same batches of urine. Each urine sample was submitted to purification with a separate IA column, and the extracts were analyzed by HPLC. Addition of BP-6-N7Gua and BP-6-N7Ade to the nonsmoker's urine was associated with the appearance of a single, prominent peak at the retention time of each adducted base (Figure 1A,C). A fraction bracketing these peaks (Figure 1A) and the same fraction taken from control, untreated urine (Figure 1C) were analyzed with a second HPLC program. Two prominent peaks exhibiting the retention times of BP-6-N7Gua and BP-6-N7Ade were associated with the HPLC fraction of treated urine but not with the same fraction of untreated urine (Figure 1B,D). The peak occurring at the retention time of BP-6-N7Gua was well-resolved (Figure 1B). However, the peak occurring at the retention time of BP-6-N7Ade (Figure 1B) was not separated from a smaller, adjoining peak (Figure 1D). The minimum recoveries of BP-6-N7Gua and BP-6-N7Ade were 85−95 and 70−85%, respectively, as determined from the AUC and comparison with standards analyzed under the same conditions of HPLC. The area of the BP-6-N7Ade peak was corrected for contamination with the closely migrating peak seen in the nonspiked sample. AUCs for BP-6-N7Ade and BP-6-N7Gua were linear from 0.1 to 2.0 pmol and 1.0 to 50 pmol, respectively. A fraction of the second HPLC separation, bracketing the retention times of BP-6-N7Gua and BP-6-N7Ade (Figure 1B,D), was extracted with $CHCl_3$ and concentrated to 20 $\mu$L in $Me_2SO$ for analysis by LC/MS and LC/MS/MS.

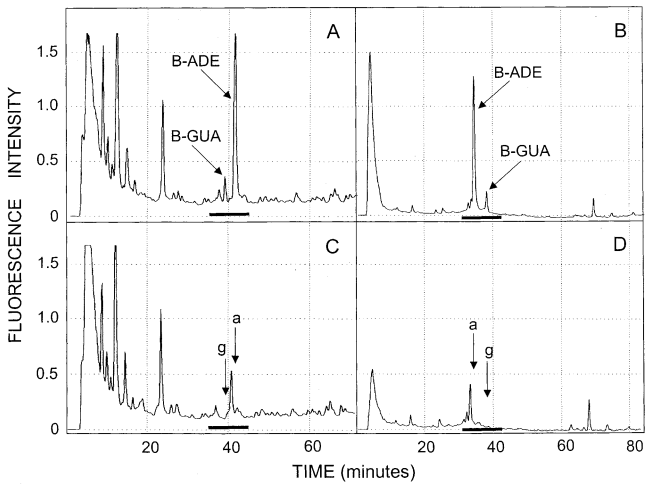


**Figure 1.** Evaluation of an IAC/HPLC protocol for extraction and purification of BP-6-N7Ade and BP-6-N7Gua added to urine collected from a nonsmoker. A mixture of 0.1 pmol of BP-6-N7Ade and 1.0 pmol of BP-6-N7Gua was added to 80 mL of urine (A and B) collected from a nonsmoker (male, age 57). Eighty milliliters of the same urine specimen was left untreated (C and D). Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-adducted bases. IAC extracts were fractionated with a 2.0 mm × 250 mm reverse phase column eluted with a $CH_3CN/H_2O$ program (A and C). Eluate was collected in an interval (solid bar) bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua. The eluate was concentrated and then fractionated with an $EtOH/CH_3CN/H_2O$ program (B and D). Two prominent peaks, at the retention times of BP-6-N7Ade and BP-6-N7Gua, were present in the treated sample (B) but not in the untreated sample (D). A peak migrating very close to the retention time of BP-6-N7Ade (D) was not separated from BP-6-N7Ade (B). Recoveries were 75% for BP-6-N7Ade and 90% for BP-6-N7Gua.

We then turned to analysis of urine from a cigarette smoker. HPLC analysis of the smoker's urine treated by addition of BP-6-N7Gua and BP-6-N7Ade revealed a prominent peak at the retention time of each adducted base (Figure 2A). These peaks were not apparent in the chromatogram of IAC-extracted, untreated urine (Figure 2C), although the latter yielded a peak with a retention

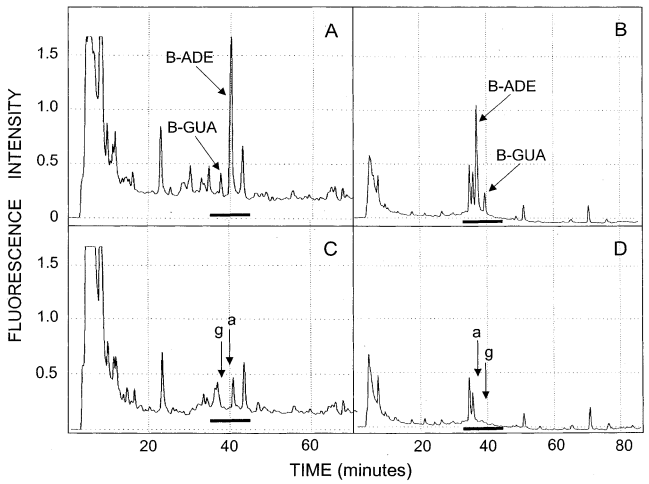


**Figure 2.** Evaluation of an IAC/HPLC protocol for extraction and purification of BP-6-N7Ade and BP-6-N7Gua added to urine collected from a cigarette smoker. A mixture of 0.1 pmol of BP-6-N7Ade and 1.0 pmol of BP-6-N7Gua was added to 80 mL of urine (A and B) collected from a cigarette smoker (male, age 46). Eighty milliliters of the same urine specimen was left untreated (C and D). Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-adducted bases. IAC extracts were fractionated with a 2.0 mm × 250 mm reverse phase column eluted with an $CH_3CN/H_2O$ program (A and C). The eluate was collected in an interval (solid bar) bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua. The eluate was concentrated and then fractionated with an $EtOH/CH_3CN/H_2O$ program (B and D). Two prominent peaks, at the retention times of BP-6-N7Ade and BP-6-N7Gua, were present in the treated sample (B) but not in the untreated sample (D). Recoveries were 70% for BP-6-N7Ade and 90% for BP-6-N7Gua.

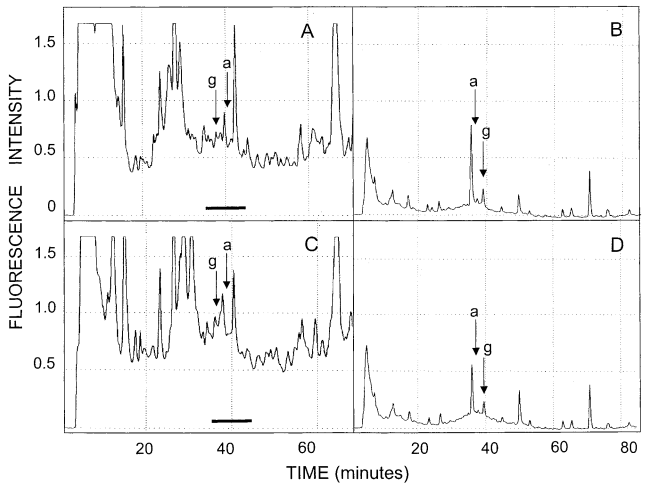


**Figure 3.** HPLC fractionation and analysis of an IAC extract of urine samples collected from Chinese women exposed to household coal smoke. Urine (15 mL) was collected from two Chinese women, ages 49 (A and B) and 44 (C and D), who were exposed to unvented, household coal smoke. Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-6-N7Gua and BP-6-N7Ade. IAC extracts were fractionated with a 2.0 mm × 250 mm reverse phase column eluted with a $CH_3CN/H_2O$ program (A and C). The eluate was collected in an interval (solid bar) bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua. The eluate was concentrated and then fractionated with an $EtOH/CH_3CN/H_2O$ program (B and D). A prominent peak, migrating at the retention time of BP-6-N7Gua, was identified in each urine sample (B and D).

time only ca. 1 min later than the expected retention time of BP-6-N7Ade. The eluates corresponding to the retention times of BP-6-N7Gua and BP-6-N7Ade were collected and analyzed with a second HPLC program. The HPLC fraction of treated sample yielded a prominent and well-resolved peak at the retention time of each adducted base (Figure 2B). These peaks were absent in the analysis of the same fraction of untreated urine (Figure 2D). Estimated minimum recoveries were the same as those reported for standards added to urine of the nonsmoker (above). A fraction of the second HPLC separation (Figure 2B,D), bracketing the retention times of BP-6-N7Gua and BP-6-N7Ade, was extracted with $CHCl_3$ and concentrated to 20 $\mu$L in $Me_2SO$ for analysis by LC/MS and LC/MS/MS.

**Analysis of Urine from Coal Smoke-Exposed Individuals: IAC Coupled with HPLC.** Coal smoke-exposed women experience a BP intake ca. 30 times that of cigarette smokers (*10*). Consequently, we anticipated more fluorescent material in a 15 mL urine sample from these women than that in an 80 mL sample from a cigarette smoker. The first HPLC analysis of urine from each of two coal smoke-exposed women yielded a complex chromatogram. Each chromatogram exhibited a small, poorly resolved peak at the retention time of BP-6-N7Gua and no peak at the retention time of BP-6-N7Ade (Figure 3A,C). Eluates that had the retention times of BP-6-N7Gua and BP-6-N7Ade (Figure 3A,C) were collected and analyzed with a second HPLC program. This fraction of each urine sample yielded a distinct peak at the retention time of BP-6-N7Gua (Figure 3B,D). Although these peaks signaled the presence of BP-6-N7Gua in the samples, they were not sufficiently resolved to permit quantification of the adducted base from its AUC. The same fraction of urine from two Chinese-American controls (Figure 4A,C) did not signal the presence of either BP-6-N7Gua or BP-6-N7Ade (Figure 4B,D). Eluates that had the elution times of the adducted bases (Figure 3B,D and Figure 4B,D) were extracted with $CHCl_3$ and concentrated to 20 $\mu$L in $Me_2SO$ for analysis by LC/MS and LC/MS/MS.

**Analysis of HPLC Fractions of IAC Extracts of Human Urine: LC/Quadrupole Ion-Trap Mass Spectrometry.** To validate our IAC/HPLC protocol and to confirm the presence of BP-6-N7Gua in HPLC fractions of IAC-extracted urine from coal smoke-exposed women, we analyzed various HPLC fractions by LC/MS and LC/MS/MS. Material present in smoker's urine spiked with 1.0 pmol of BP-6-N7Gua and eluting from 23.91 to 26.26 min produced a product ion spectrum (Figure 5A) showing clearly the *m*/*z* 252, 360, 378, and 385 product ions, which are characteristic of BP-6-N7Gua (Figure 5C). A sample of the same urine that was not spiked did not produce the characteristic product ion spectrum of BP-6-N7Gua (Figure 5B), and the adducted base was considered "not detected". BP-6-N7Gua was estimated to be 0.5 pmol in the original urine sample as determined from the time-integrated signal of its *m*/*z* 252 product ion by LC/MS/MS. The same analysis of the nonsmoker's urine spiked with 1 pmol of BP-6-N7Gua (data not shown) showed a level of 1 pmol of the adducted base in the original urine sample. In the absence of an internal standard, these quantitative estimates must be considered minimum estimates. Nevertheless, they are in agreement with estimates based on HPLC analysis (above). BP-6-N7Ade was present below the detection limit of the mass spectrometer.

Material present in the urine of the coal smoke-exposed women and eluting from 24.76 to 26.05 min and 24.43 to

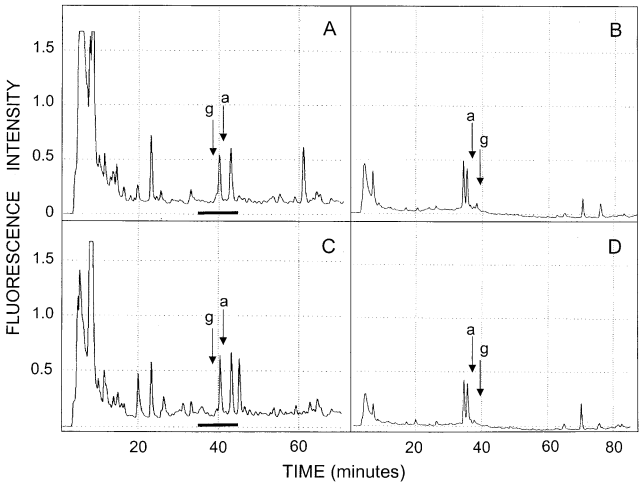


**Figure 4.** HPLC fractionation and analysis of an IAC extract of urine samples collected from Chinese-American women exposed neither to cigarette smoke nor to coal smoke. Twenty milliliter samples of urine were collected from two Chinese-American women, ages 36 (A and B) and 54 (C and D), who were exposed to neither cigarette smoke nor household coal smoke. Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-6-N7Gua and BP-6-N7Ade. IAC extracts were fractionated with a 2.0 mm × 250 mm reverse phase column eluted with a $CH_3CN/H_2O$ program (A and C). Eluate was collected in an interval (solid bar) bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua. The eluate was concentrated and then fractionated with an $EtOH/CH_3CN/H_2O$ program (B and D). Both urine samples were characterized by the absence of fluorescence peaks at the retention times of BP-6-N7Ade and BP-6-N7Gua (B and D).

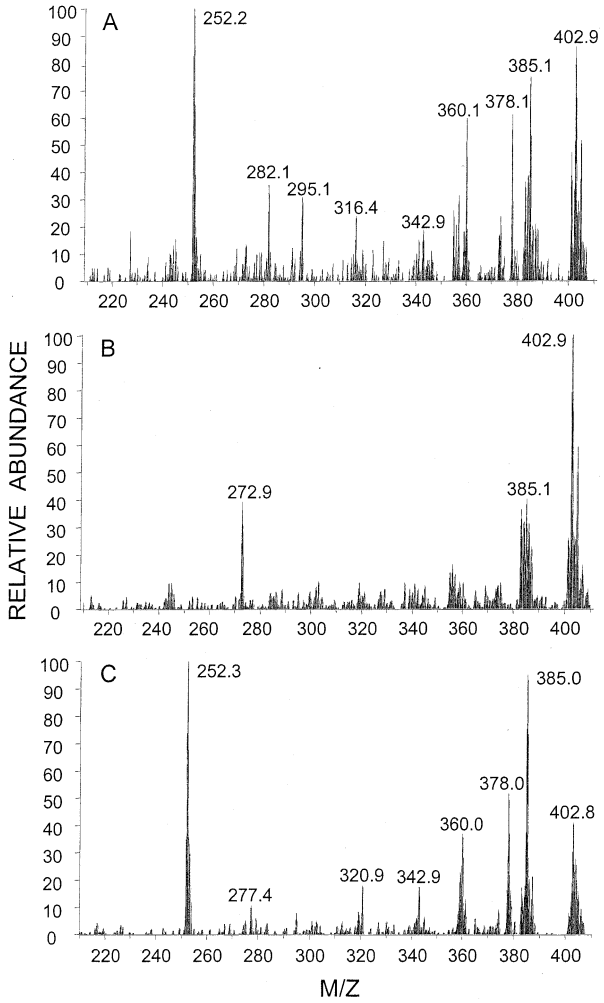


**Figure 5.** LC/MS/MS confirming IAC extraction of BP-6-N7Gua added to urine of a cigarette smoker. Urine (160 mL) collected from a cigarette smoker (male, age 46) was split into two equal volumes. One volume was treated by addition of 0.1 pmol of BP-6-N7Ade and 1.0 pmol of BP-6-N7Gua (A), and one volume was left untreated (B). Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-adducted bases. IAC extracts were fractionated by reverse phase HPLC. The eluate collected in an interval bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua was extracted, concentrated, and then analyzed by LC/MS/MS. The BP-6-N7Gua standard was analyzed directly by MS/MS (C). Product ion spectra indicate the presence of quantifiable BP-6-N7Gua in the IAC extract of urine treated by addition of the adducted bases.

26.45 min, respectively, produced product ion spectra (Figure 6A,B) that included the *m*/*z* 252, 360, 378, and 385 product ions, which are characteristic of BP-6-N7Gua (Figure 5C), and confirmed the presence of adducted base in the urine samples. Urine samples collected from nonexposed women yielded product ion spectra (Figure 6C,D) that were not characteristic of BP-6-N7Gua (Figure 5C) and, consequently, were viewed to contain no detectable BP-adducted base. BP-6-N7Gua was quantified by LC/MS/MS from the time-integrated signal of its *m*/*z* 252 product ion and estimated to be present in the urine of coal smoke-exposed women at 20 and 50 fmol per mg of urinary creatinine. Again, in the absence of an internal standard, these must be considered minimum estimates.

## Discussion

The present study demonstrates efficient and highly selective extraction of BP-adducted DNA bases from human urine, with a rapid and simple IAC protocol. Urine samples of up to 80 mL were passed through an IA column prepared with a Mab (*14*) of high specificity and affinity for the BP-adducted DNA bases, BP-6-N7Gua and BP-6-N7Ade. The latter represent 32% of all DNA adducts formed by metabolites of BP (*8*). Captured bases were eluted with acetonitrile and then analyzed directly by analytical HPLC with fluorescence detection, followed by LC/MS/MS. Our IAC protocol afforded a remarkable improvement in extraction speed and selectivity, in comparison to Sep-Pak extraction used in our first demonstration of BP-adducted DNA bases in urine of cigarette smokers and Chinese women exposed to coal smoke (*10*). In the latter study, Sep-Pak extracts were shown to contain abundant fluorescent material as indicated by preparative (10 mm × 250 mm column) HPLC chromatograms exhibiting a continuum of strong fluorescence signals. Comparison of these chromatograms with analytical (2 mm × 250 mm column) HPLC chromatograms produced with IAC extracts of similar quantities of urine indicates ca. a 25-fold reduction of fluorescent material in the IAC extracts. Marked reduction of fluorescent material in the IAC extracts was associated with efficient recovery of both BP-6-N7Gua and BP-6-N7Ade added to urine samples. Importantly, amounts of the adducted bases added to urine were comparable to amounts detected in the urine of some cigarette smokers and women exposed to coal smoke (*10*). Thus, the use and efficiency of our IAC protocol apply to environmental exposures.

The degree of complexity of the chromatograms produced with IAC extracts of urine was due primarily to

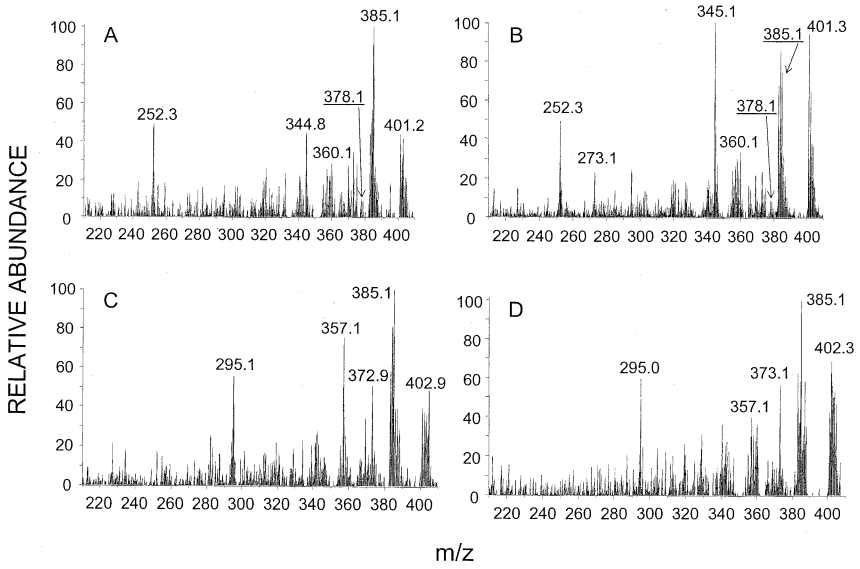


**Figure 6.** Analysis of fractions from IAC/HPLC of urine from coal smoke-exposed individuals, by LC/MS/MS, demonstrating the presence of BP-6-N7Gua. Urine (15 mL) was collected from two Chinese women, ages 49 (A) and 44 (B), who were exposed to unvented, household coal smoke. Control urine samples were collected from two Chinese-American women, ages 36 (C) and 54 (D), who were exposed neither to cigarette smoke nor to household coal smoke. Each sample was extracted with a separate IA column incorporating a mouse Mab with high specificity and affinity for both BP-6-N7Gua and BP-6-N7Ade. IAC extracts were fractionated by reverse phase HPLC. Eluate collected in an interval bracketing the retention times of BP-6-N7Ade and BP-6-N7Gua was extracted, concentrated, and then analyzed by LC/MS/MS. The product ion spectra indicated the presence of BP-6-N7Gua (*m/z* 252, 360, and 385) in urine samples from the coal smoke-exposed women (A and B) but not in the urine of the Chinese-American controls (C and D).

nonspecific adhesion of urinary constituents to the glass bead matrix, rather than to cross-reactivity of these constituents with our Mab. Urine extracted with glass beads lacking a Mab coat yielded similarly complex chromatograms (data not shown). In addition, only ca. 5% of added BP-6-N7Gua was recovered from a nonsmoker's urine extracted with glass beads lacking Mab (data not shown). A significant improvement in IAC extraction may be achieved by coupling our Mab to nonporous beads with a high density of polar groups, thereby reducing hydrophobic contaminants extracted with the BP-adducted DNA bases. This modification could eliminate contaminants eluting close to BP-6-N7Gua and BP-6-N7Ade during HPLC and, consequently, both lower detection limits and improve quantification of the adducted bases.

BP-6-N7Gua and BP-6-N7Ade, at 1.0 and 0.1 pmol per 80 mL of urine, respectively, were efficiently extracted by IAC and readily detected by analytical HPLC coupled with fluorescence detection. Under the conditions of our study, BP-6-N7Ade produced a greater molar fluorescence, in comparison to BP-6-N7Gua. This is consistent with the mass spectral analyses of IAC/HPLC fractions containing the adducted bases. Detection limits, established using the characteristic fragments of BP-6-N7Gua and BP-6-N7Ade, were similar. The product ion spectrum of BP-6-N7Gua was readily seen for IAC/HPLC fractions of urine containing 1.0 pmol of the adducted base. In contrast, the product ion spectrum of BP-6-N7Ade could not be seen for fractions prepared with 80 mL of urine containing 0.1 pmol of the adducted base. The detection limit (ca. 5−10 fmol per mL of urine sample) of our combined IAC/HPLC/MS protocol must be lowered by ca. 50-fold for routine quantification of BP-adducted DNA bases in urine of cigarette smokers (*10*). Use of a smaller bore analytical column for the second HPLC fractionation is feasible and, in conjunction with reduced contamination of IAC extracts, would significantly improve detection by fluorescence emission. Improved detection of BP-adducted DNA bases by LC/MS/MS may be achieved with the use of a smaller bore column linked to the mass spectrometer. The fused silica capillary column used in this study has an internal diameter of 0.3 mm and operates at a flow rate of 5 $\mu$L per min. A capillary of 0.1 mm diameter would produce ca. a 10-fold concentration of analyte and would operate at a flow rate of 500 nL/min. Thus, the same quantity of analyte present in 5 $\mu$L of eluate from a 0.3 mm capillary would be present in 500 nL of eluate from a 0.1 mm capillary. The combination of doubling or tripling the volume of urine extracted by IAC and reducing diameter of the capillary column will permit measurement of BP-adducted DNA bases at the desired level (ca. 0.1−0.2 fmol per mL of urine sample).

Failure to detect BP-adducted DNA bases in the urine of cigarette smokers was not caused by insufficient capacity of the IA columns for capture of BP-adducted DNA bases, in the presence of cross-reactive contaminants (*14*). Assuming that a moderate smoker takes in ca. 800 ng (3.2 nmol) BP per day, we estimate that this would produce a total of ca. 1−2 pmols of urinary BP-6-N7Gua plus BP-6-N7Ade (*15*). A 100 mL sample of urine would contain ca. 1/15 (daily urinary output is ca. 1500 mL) of these quantities or ca. 65−130 fmol of the adducted bases. Our IA columns consist of 10−12 mg of Mab bound to ca. 3 g of glass beads, corresponding to ca. 50−60 nmol of binding sites available to trap the adducted bases. Immunochemical studies of the binding of Gua, guanosine, BP metabolites, BP, and other PAH to Mab CB53 (*14*) demonstrated that most of these compounds exhibited binding affinities 2−5 orders of magnitude lower than those of BP-6-N7Gua and BP-6-N7Ade. BP-7,8-dihydrodiol exhibited the highest cross-reactivity, with a binding affinity ca. 10 times lower than that of BP-6-N7Gua or BP-6-N7Ade. Thus, the binding capacity of our IA columns greatly exceeds the expected quantity

of BP-6-N7Gua, BP-6-N7Ade, and potential cross-reactants. This conclusion is supported by the present work indicating similar recoveries of BP-6-N7Ade and BP-6-N7Gua from PAH-contaminated urine of smokers and relatively clean urine of nonsmokers.

Our present work is aimed at development of an IAC-based protocol with efficiency similar to ELISA-based quantification of stable PAH−DNA adducts, for quantification of depurinated BP-adducted DNA bases in the urine of cigarette smokers and others exposed to PAH. We propose that urinary concentration of these adducted bases in combination with (i) leukocyte concentrations of stable PAH−DNA bases and (ii) DNA repair phenotype may provide an accurate predictor of individual risk of PAH-associated cancer. Results of the present study support further development of our IAC-based protocol for detection and quantification of BP-6-N7Ade and BP-6-N7Gua in the urine of PAH-exposed individuals.

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