

Analysis of 4-Aminobiphenyl–DNA Adducts in Human Urinary Bladder and Lung by Alkaline Hydrolysis and Negative Ion Gas Chromatography–Mass Spectrometry

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Analysis of carcinogen–DNA adducts has been regarded as a useful means of assessing human exposure to chemical carcinogens. We have established a method for quantitation of 4-aminobiphenyl (4-ABP)–DNA adducts by alkaline hydrolysis and gas chromatography with negative ion chemical ionization mass spectrometry (GC–NICI–MS). Aliquots of DNA (typically 100 µg/ml) were spiked with an internal standard, d₉–4-ABP, and were hydrolyzed in 0.05 N NaOH at 130°C overnight. The liberated 4-ABP was extracted with hexane and derivatized using pentafluoropropionic anhydride in trimethylamine for 30 min at room temperature prior to GC–NICI–MS. With *in vitro* [³H]N-hydroxy-4-ABP modified DNA standards, we observed 59 ± 7% (*n* = 9) recovery of the 4-ABP and a linear correlation between hydrolyzed 4-ABP and the adduct levels ranging from about 1 in 10⁸ to 1 in 10⁴ nucleotides (*r* = 0.999, *n* = 9). The method was further validated by comparison of the results with that obtained by the ³²P-postlabeling method. There was excellent agreement (*r* = 0.994, *p* < 0.001) between the two methods for quantitation of the adduct in eight samples of *Salmonella typhimurium* DNA treated with 4-ABP and rat liver S9, although the ³²P-postlabeling method gave slightly higher values. The DNA adducts in 11 human lung and 8 urinary bladder mucosa specimens were then determined by our GC–NICI–MS method. The adduct levels were found to be <0.32 to 49.5 adducts per 10⁸ nucleotides in the lungs and <0.32 to 3.94 adducts per 10⁸ nucleotides in the bladder samples. Our results indicate that the alkaline hydrolysis/GC–NICI–MS method is sensitive, structure-selective, and accurate, and will be useful for molecular dosimetry of human exposure to this carcinogen. — Environ Health Perspect 102(Suppl 6):11–16 (1994)

Key words: 4-aminobiphenyl, urinary bladder cancer, lung cancer, DNA adducts, GC–NICI–MS

Introduction

The covalent modification of DNA by chemical carcinogens appears to be a critical event in chemical carcinogenesis. As a result, the analysis of carcinogen–DNA adducts has been considered to be a useful means of assessing human exposure to chemical carcinogens. 4-Aminobiphenyl (4-ABP) is a potent human urinary bladder carcinogen and is present in significant quantities in tobacco smoke (1). This aromatic amine is metabolically activated to several electrophilic intermediates, which then covalently bind to cell macromolecules. The reaction of activated 4-ABP with DNA *in vitro* and *in vivo* in laboratory ani-

mals is known to result in formation of three to four nucleoside adducts. The predominant adduct is *N*-(deoxyguanosin-8-yl)-ABP (dG-C8-ABP), which accounts for about 70% of the bound radioactivity (2,3). Evidence for human exposure to 4-ABP and its metabolic activation was initially obtained from the detection of 4-ABP-hemoglobin adducts in humans, with higher levels being observed in cigarette smokers versus nonsmokers (4–6) and in patients with a history of urinary bladder cancer versus controls (7).

With recent advances in analytical methodology, 4-ABP–DNA adducts have been detected either by immunochemical or ³²P-postlabeling–HPLC techniques in human lung or urinary bladder at levels ranging from one adduct per 10⁶ to one adduct per 10⁹ nucleotides (8,9). More recently, 4-ABP–DNA adducts were found as smoking related adducts in human urinary bladder biopsies using ³²P-postlabeling

methods (10). These findings suggested that 4-ABP is likely to be an important factor in the etiology of tobacco-related human cancers. However, since these methods may not always provide sufficient specificity and precision, a sensitive, structure-specific, and relatively simple method was warranted for molecular dosimetry of human exposure to this carcinogen. Furthermore, a specific methodology for confirmation of sample analyses was also highly desirable.

Previous studies have shown that C8-deoxyguanosine adducts of arylamines 2-amino-6-methylpyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) (11) and 2-aminofluorene (12), are susceptible to hydrolysis in sodium hydroxide or hydrazine to release parent arylamines, respectively. These studies encouraged us to investigate 4-ABP-adducted DNA. In this article, we report the development of a method for quantitation of 4-ABP–DNA adducts by alkaline hydrolysis and gas chromatography with negative ion

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chemical ionization mass spectrometry (GC-NICI-MS) analysis.

Materials and Methods

Chemicals

[2,2'-³H]*N*-Hydroxy-4-ABP (91.2 mCi/mmoles) and unlabeled 4-ABP were acquired from Chemsyn Science Laboratories (Lenexa, KS) and Aldrich Chemical Co. (Milwaukee, WI), respectively. All aqueous solutions for GC-MS analysis were prepared with water distilled from a dilute solution of KMnO₄. Hexane (Resi-analyzed grade) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Trimethylamine in hexane was prepared by adding 1 g reagent-grade trimethylamine hydrochloride from Aldrich Chemical Company, Inc. (Milwaukee, WI) to 2 ml water, neutralizing with NaOH and extracting into 5 ml hexane. Pentafluoropropionic anhydride (PFPA), purchased from Pierce (Rockford, IL), was stored in air-tight vials and replaced periodically. The internal standard, d₉-4-ABP, was a gift of SR Tannenbaum (MIT Cambridge, MA). A stock solution of d₉-4-ABP in methanol (20 µg/ml) was prepared and stored at -20°C. Various dilutions were made on each day of use. All the enzymes for ³²P-postlabeling assay were obtained from Sigma Chemical Co. (St. Louis, MO), except T₄ polynucleotide kinase, which was from U.S. Biochemical Corp. (Cleveland, OH). [γ-³²P]ATP (7000 Ci/mmole) was obtained from ICN Radiochemicals (Irvine, CA). PEI-cellulose thin-layer plates, manufactured by Macherey-Nagel (no. 801053), were purchased from Alltech Associates, Inc. (Deerfield, IL). X-ray film was from Eastman-Kodak (Dallas, TX). All other chemicals were of the highest grade available.

Preparation of DNA Samples

[³H]*N*-Hydroxy-4-ABP modified DNA standard was prepared by the reaction of radiolabeled *N*-hydroxy-4-ABP with calf thymus DNA in 100 mM potassium citrate/0.1 mM EDTA, pH 5, as described previously (2,3). The adduct levels were determined by enzymatic hydrolysis, HPLC, and scintillation counting. The *Salmonella typhimurium* TA1538 and TA98 DNA was isolated from the bacteria treated with various concentrations of 4-ABP ranging from 5 to 24 µg/ml in the presence of Aroclor-treated rat liver S9 under the conditions described (13). The bacteria were digested for 10 min with 0.1% SDS, 50 mM Tris-HCl buffer (pH 7.9), 10 mM EDTA,

and 0.28 mg/ml proteinase K; DNA was purified from the lysates as described (14). Human lung and urinary bladder tissues obtained by surgery or autopsy were from the John A. McClellan Memorial Veterans Hospital in Little Rock, AR. The DNA was isolated by homogenization of the tissue, lysis of the cells with SDS, digestion with protease, and extraction with phenol and chloroform/isoamyl alcohol; traces of RNA were removed by digestion with RNase and DNA was precipitated with ethanol (15). The smoking histories of the lung donors are shown in Table 1; however, the smoking histories of the urinary bladder donors were not available.

Alkaline Hydrolysis of 4-ABP from DNA

Aliquots of DNA solution (typically 100 µg DNA/ml) in screw-capped tubes (13 × 100 mm) were brought to 0.05 N with respect to NaOH and extracted three times with 2 ml of hexane before spiking with internal standard, d₉-4-ABP, (2 to 200 pg in 10 µl methanol, for GC-NICI-MS analysis) or 4-ABP (as UV marker for HPLC analysis). The solution was then heated at 130°C overnight (18 hr) in a Temp-Blok module heater (Lab-Line Instruments Inc., Melrose, IL) that had been filled with sea sand. The samples were then cooled to room temperature and the released 4-ABP was extracted into hexane (2 ml, twice). For HPLC analysis, [³H]*N*-hydroxy-ABP-modified DNA was hydrolyzed. After extraction, hexane was removed and the residue redissolved in methanol. The 4-ABP was separated by HPLC on a Waters µBondapak C₁₈ column (3.9 × 300 mm). Elution was carried out with a linear gradient starting from 20% methanol in water to 100% methanol over 25 min at a flow rate of 2 ml/min. Under these conditions, the retention time of 4-ABP was 15.3 min. The eluate was collected at 1-min intervals and mixed with scintillation fluid for counting radioactivity in a Tracor Model 688 Analytic Marker III scintillation counter (Elk Grove, IL). For GC-NICI-MS analysis, the extracted 4-ABP and the internal standard were derivatized as described below.

Derivatization of 4-ABP

4-ABP was derivatized on the basis of the procedure described previously (4). Briefly, 10 µl of trimethylamine in hexane was added to the combined hexane extracts obtained as described above in a test tube, followed by 10 µl of PFPA. After reaction for 30 min at room temperature, the solvent and excess reagents were removed by vacuum

Table 1. 4-ABP-DNA adducts in human lung tissues.

Donor no.	Smoking history	4-ABP adducts ^a
1	1.5 packs/day for 5 year	3.68
2	1.5-2 packs/day	7.52
3	2 packs/day	1.54
4	Not available	4.26
5	Ex-smoker (0.5 pack/day)	17.60
6	Nonsmoker	1.41
7	1-2 packs/day for 2 year	7.55
8	1 pack/day for 66 yr	49.50
9	1-1.5 packs/day for 55 year	ND ^b
10	Not available	4.26
11	Not available	6.37

^aValues represent the number of dG-C8-ABP adducts per 10⁸ nucleotides. ^bNot detected. The adduct level < 0.32 adduct per 10⁸ nucleotides.

centrifugation. The PFPA derivative of 4-ABP was redissolved in an appropriate amount of hexane before injection to GC-NICI-MS.

Gas Chromatography-Mass Spectrometry

GC-NICI-MS analyses were carried out on a Varian 3400 gas chromatograph with a SPI injector interfaced (225°C) to a Finnigan 4000 mass spectrometer system (Finnigan MAT, San Jose, CA). Chromatographic separation was achieved using a DB5 fused silica capillary column (32 m × 0.25 mm id). Helium was used as carrier gas at a head pressure of 20 psi. The column oven was heated from 60 to 180°C at 20°C/min and then at 10°C/min to 250°C. Under these conditions, the retention times of the PFPA derivatives of 4-ABP and d₉-4-ABP were about 7 min. The mass spectrometer was operated in the negative ion chemical ionization (NICI) mode with an electron energy of 100 eV. Methane gas was admitted to an indicated ion source pressure of about 0.25 torr and the source temperature was maintained at 220°C. Quantification was accomplished using selected ion monitoring (SIM) of the (M-HF)⁻ ions of the PFPA derivatives of 4-ABP (m/z 295) and d₉-4-ABP (m/z 304). Data were processed by an INCOS data system using IDOS 2 software.

Calculations

The integrated peak area of the 295 ion was divided by the peak area of the 304 ion and then multiplied by the ratio of the molecular weights of the respective compounds (169/178) and by the amount of internal standard added to determine the amount (in picograms) of 4-ABP in the sample. This value was corrected by the recovery, divided by the amount in micrograms of

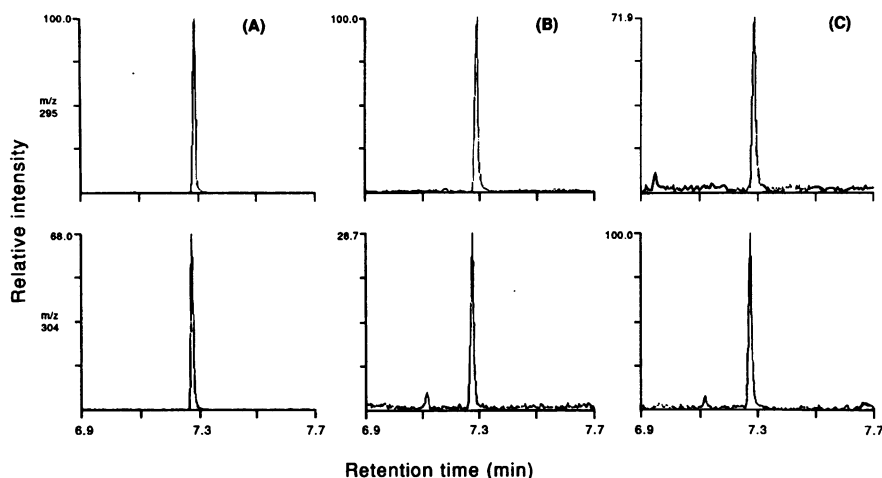


Figure 1. SIM chromatograms for the analysis of 4-ABP (m/z 295) and d_5 -4-ABP (m/z 304). The traces were produced from samples: (A) [^3H]N-hydroxy-ABP-modified DNA containing 8.4 adducts/ 10^5 nucleotides; (B) human lung DNA no. 7; and (C) human urinary bladder DNA no. 2.

DNA in the sample and by the molecular weight of 4-ABP to calculate the binding level, expressed as pmole 4-ABP per mg DNA or as adducts/ 10^6 nucleotides.

^{32}P -Postlabeling Determination of dG-C8-ABP

^{32}P -Postlabeling assays were carried out essentially based on the method described by Gupta et al. (16). Briefly, the DNA samples and [^3H]N-hydroxy-4-ABP-modified DNA standards (each 5 μg DNA) were hydrolyzed to 3'-nucleotides with 0.2 unit of micrococcal endonuclease and 2.5 μg of spleen phosphodiesterase by incubation at 37°C for 3 hr. Adducted nucleotides were enriched by *n*-butanol extraction (17). Samples were then [$5'$ - ^{32}P]phosphorylated at 37°C for 40 min using 3 units of polynucleotide kinase and 200 μCi of [γ - ^{32}P]ATP. Eight microliters of the labeled mixture (2 μg DNA) was spotted in duplicate on PEI-cellulose sheets and developed in four directions (16,17) with buffers as follows: D1 and D2 buffers consisted of 1.7 M sodium phosphate, pH 6.0; D3 and D4 buffers contained 3.6 M lithium formate/8.5 M urea (pH 3.5) and 1.2 M lithium chloride/0.5 M Tris-HCl/8.0 M urea (pH 8.0), respectively. The adducts were located by autoradiography and the corresponding area of the dG-C8-ABP on the sheets was excised and the ^{32}P radioactivity determined by Cerenkov counting. The adduct levels were quantitated by comparing the radioactivity in the spot to

the corresponding spot from the parallel analysis of [^3H]N-hydroxy-4-ABP-modified DNA standards. Using the adduct levels from about 1 in 10^8 to 1 in 10^5 nucleotides, the standard curve was linear with a correlation coefficient of 0.999.

Results

Hydrolysis Conditions

Preliminary experiments were performed to determine the yield of 4-ABP hydrolyzed from dG-C8-ABP and 4-ABP-adducted DNA and to optimize the hydrolysis conditions. [^3H]N-Hydroxy-ABP-modified DNA (100 $\mu\text{g}/\text{ml}$) and [^3H]dG-C8-ABP (10 μM) were hydrolyzed using a variety of reaction conditions. These included varying the concentration of hydrazine hydrate or NaOH from 0.01 to 1 N and incubation temperature from room temperature to 130°C. It was found that the optimal conditions were 0.05 N NaOH overnight at 130°C, although similar results could be achieved by hydrazinolysis. HPLC analysis showed that 55 and 85% of 4-ABP were hydrolyzed from [^3H]N-hydroxy-ABP-modified DNA and [^3H]dG-C8-ABP, respectively (data not shown). Increasing the NaOH concentration to 1 N did not increase the yield further. The recovery of the product, 4-ABP, subjected to hydrolysis under these conditions was $88 \pm 3\%$ ($n=3$). These hydrolysis conditions were then employed in the further experiments with the GC-NICI-MS analysis used in this study.

Validation of the Method

Alkaline hydrolysis of 4-ABP from the 4-ABP-adducted DNA and subsequent GC-NICI-MS analysis was validated using *in vitro* [^3H]N-hydroxy-ABP-modified calf thymus DNA in which the adduct levels were known by measuring the radioactivity of the bound [^3H]4-ABP residues, and by comparison of this method to the ^{32}P -post-labeling method for quantitation of dG-C8-ABP adducts in the DNA isolated from *S. typhimurium* strains TA1538 and TA98, which were treated with various concentrations of 4-ABP under the conditions used for mutagenicity assays as described in Materials and Methods.

[^3H]4-ABP-adducted DNA with levels from one adduct per 10^8 to one adduct per 10^4 nucleotides was analyzed by the method. A representative chromatogram of one of these samples is shown in Figure 1A. The yield of 4-ABP from the 4-ABP-adducted DNA was found to be $59 \pm 7\%$ ($n=9$) and this result was consistent with that obtained by HPLC analysis. An excellent correlation ($r = 0.999$, $n = 9$) was observed between hydrolyzed 4-ABP amounts and the adduct levels in the DNA (Figure 2). The detection limit is 10 fmoles 4-ABP per mg DNA (0.32 adduct per 10^8 nucleotides) due to the amount of about 0.006 μg of 4-ABP present in the blank. Reproducibility was assessed by sampling in triplicate for nine different samples, and the coefficient of variation was estimated to be 9.7%.

To validate this method for DNA with unknown levels of the adduct, eight DNA samples, isolated from *S. typhimurium* strains TA1538 and TA98 incubated with

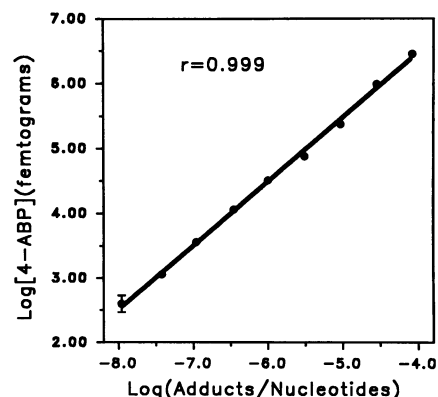


Figure 2. Standard curve for alkaline hydrolysis and GC-NICI-MS of 4-ABP adducts from *in vitro* [^3H]N-hydroxy-ABP-modified DNA. Each point represents mean value \pm SE from three analyses in different batches. In most cases error bars are within the symbols.

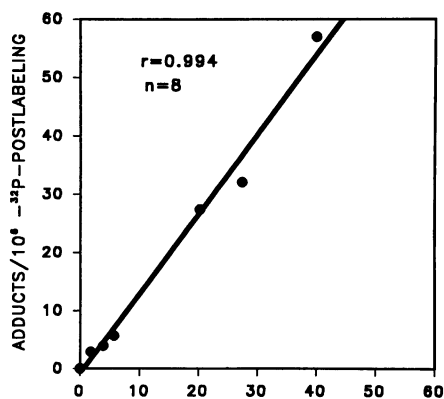


Figure 3. Correlation of 4-ABP-adduct analysis obtained by either alkaline hydrolysis and GC-NICI-MS or ^{32}P -postlabeling.

different concentrations of 4-ABP under the conditions used for determination of its S9-mediated mutagenicity, were analyzed by GC-NICI-MS and by the ^{32}P -postlabeling technique. There was complete agreement between the two methods, although the ^{32}P -postlabeling method gave slightly higher values, especially at high adduct levels (Figure 3). The correlation coefficient was 0.994 ($p < 0.001$). These results indicate that the alkaline hydrolysis and GC-NICI-MS analysis method is reliable and it is comparable to ^{32}P -postlabeling technique for quantitation of dG-C8-ABP at the adduct levels analyzed. Typical autoradiograms of ^{32}P -postlabeled [^3H]N-hydroxy-ABP-modified DNA and DNA isolated from *S. typhimurium* are shown in Figure 4.

Application of the Method to Human Tissue DNA

Eleven human lung and eight human urinary bladder mucosa DNA samples were analyzed for dG-C8-ABP adducts. The results, summarized in Table 1 and Figure 5, indicate that 10 of 11 lung DNA samples and 5 of 8 urinary bladder mucosa DNA samples were positive. The GC-NICI-MS traces of one of these positive DNA samples are shown in Figure 1B and 1C, respectively. The adduct levels in lung DNA varied among the individuals, ranging from < 0.32 to 49.5 adducts in 10^8 nucleotides. However, the adduct levels did not seem to be directly related to the numbers of cigarettes smoked per day or the duration of smoking (Table 1). The adduct levels in the eight urinary bladder mucosa DNA ranged from < 0.32 to 3.9 adducts in 10^8 nucleotides (Figure 5), indicating that the adduct levels are comparable in both the lungs and the urinary bladder.

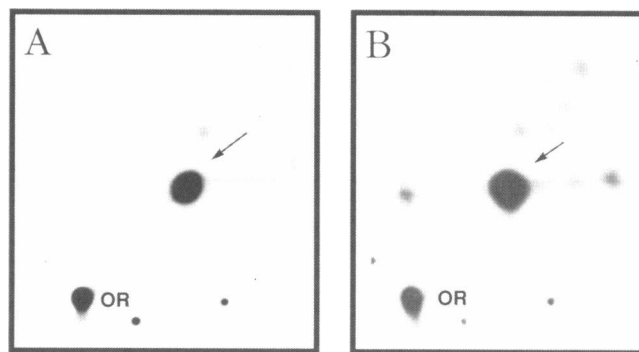


Figure 4. Autoradiograms of 4-ABP-DNA adducts analyzed by ^{32}P -postlabeling. (A) [^3H]N-hydroxy-ABP-modified DNA standard and (B) DNA from *S. typhimurium* strains TA1538 and TA98 treated with 4-ABP ("Materials and Methods"). The dG-C8-ABP spot is marked with arrow.

Discussion

Based on the previous work on the hydrolysis of carcinogenic arylamine-nucleoside adducts (11,12), we have investigated the alkaline hydrolysis of 4-ABP-modified DNA. We found that 59% of 4-ABP bound to DNA could be liberated by hydrolysis with 0.05 N NaOH at 130°C overnight. The efficiency of the hydrolysis of the adducts and the minimal interference in the samples, as seen in Figure 1, has enabled application of the GC-NICI-MS method to quantitate trace amounts of 4-ABP hydrolyzed by the procedure. With [^3H]N-hydroxy-ABP-modified DNA standards, the method is sensitive to levels as low as 10 fmole 4-ABP per mg DNA (0.32 adduct/ 10^3 nucleotides using 100 μg DNA). The method has been used to quantify adduct levels up to one adduct per 10^4 nucleotides, and the curve is linear in this range ($r = 0.999$, Figure 2). These results indicate that this method provides both high selectivity and sensitivity required for detection and quantification of the adduct at levels found in human biological samples (10,18). We also compared this GC-NICI-MS method to the most widely used ^{32}P -postlabeling technique in analysis of dG-C8-ABP in the same DNA samples. The results show that these two methods are highly correlated, although the ^{32}P -postlabeling method gave slightly higher absolute values (Figure 3). This suggests that the GC-NICI-MS method is reliable and comparable to the ^{32}P -postlabeling method for the determination of 4-ABP-DNA adducts at the levels analyzed.

Tobacco smoking is implicated as an etiologic factor in human lung and urinary bladder cancers (19-22). 4-ABP, among other aromatic carcinogens in tobacco smoke, is likely to play a role in these dis-

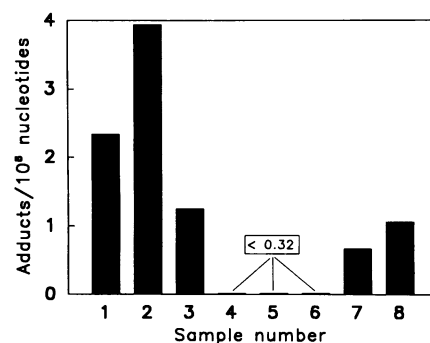


Figure 5. 4-ABP-DNA adducts in human urinary bladder mucosa.

eases because 4-ABP-DNA adducts were detected in smokers' lungs and urinary bladder (8-10). Using the GC-NICI-MS method, we have confirmed these previous findings. The adduct was found in almost all lungs and five of eight of the urinary bladders and the levels in both lungs and urinary bladder were comparable. Lack of correlation of the adduct levels in smokers' lungs to the number of cigarettes smoked may reflect limited sample size as well as the differences in actual intake of 4-ABP, which can be affected by smoking habits, such as brand of cigarette used (1,23) and the extent of smoke inhalation. Moreover, the biologic uptake and distribution of the carcinogens, metabolic phenotype, and DNA repair mechanisms are well known to vary greatly from individual to individual (23,24), and these factors undoubtedly contributed to the variations in the adduct formation. The differences in the sampling of the lung tissue also would result in variations of the DNA adduct levels because biochemical processes of the adduct formation are dependent on cell type, the anatomic location of the cells, and differentiated state

of the cells (25,26). These lung tissue specimens were not sampled from the same anatomic location. Therefore, the result does not necessarily mean that the adducts are not relevant to cigarette smoke.

4-ABP-DNA adducts also can be quantitated by the ³²P-postlabeling method and immunochemical assays (9,10). While immunochemical assays may provide sensitivity required for human biomonitoring, the accuracy of the method can be compromised by cross-reactivity of the antibodies with unknown contaminants. The ³²P-postlabeling technique is a widely used method and has been applied to analysis of 4-ABP-DNA adducts in human DNA in several studies carried out in this laboratory

and others (10,18). This method is highly sensitive and requires only low microgram amounts of DNA for analysis. However, the quantitation is compromised when the authentic adduct is not available for use as a chromatographic standard, and accurate measurements are difficult when it is applied to human tissue DNA, especially smokers' lung DNA, because the 4-ABP-adduct spots are usually located in the diagonal zone of radioactivity. Attempts were made in the current study to correlate the ³²P-postlabeling method to the GC-NICI-MS method for quantitation of 4-ABP adducts in human urinary bladder DNA, but it was not successful due to the difficulty in precisely measuring ³²P

radioactivity in half the cases in which the adduct spots were located in the diagonal zone (data not shown). Under our experimental conditions, however, the GC-NICI-MS method obviated this problem for the adduct analysis and offered not only relatively high selectivity and sensitivity but also accuracy.

In conclusion, we have shown that 4-ABP-DNA adducts can be detected reliably by alkaline hydrolysis and GC-NICI-MS analysis. This method is sensitive, structure-selective, and appears to be useful for molecular dosimetry of human exposure to the carcinogen 4-ABP.

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