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Quantification of 3-Nitrobenzanthrone-DNA Adducts Using Online Column-Switching HPLC-Electrospray Tandem Mass Spectrometry

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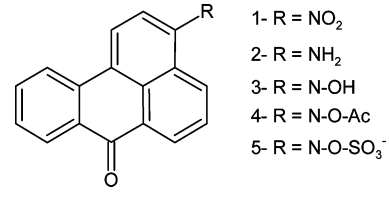
The aromatic nitroketone 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one; 3-NBA) is an extremely potent mutagen and a suspected human carcinogen detected in the exhaust of diesel engines and in airborne particulate matter. 3-NBA is metabolically activated via reduction of the nitro group to the hydroxylamine (*N*-OH-3-ABA) to form covalent DNA adducts. Thus far, the detection and quantification of covalent 3-NBA-DNA adducts has relied solely on ^{32}P-postlabeling methodologies. In order to expand the range of available techniques for the detection and improved quantification of 3-NBA-DNA adducts, we have developed a method based upon online column-switching HPLC coupled to electrospray tandem mass spectrometry, with isotopic dilution of ^{15}N-labeled internal standards. This methodology was applied to the determination of three 3-NBA-derived adducts: 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA), *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA) and 2-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone (dG-C8-C2-3-ABA). Dose-dependent increases were observed for all three adducts when salmon testis DNA was reacted with *N*-acetoxy-3-aminobenzanthrone (*N*-AcO-3-ABA). dG-C8-C2-3-ABA was detected at much lower levels (overall 1%) than the other two adducts. DNA samples isolated from tissues of rats treated either intratracheally with 3-NBA or intraperitoneally with *N*-OH-3-ABA were analyzed by mass spectrometry, and the results compared to those obtained by ^{32}P-postlabeling. The method required 50 μg of hydrolyzed animal DNA on column and the limit of detection was 2.0 fmol for each adduct. dG-C8-C2-3-ABA was not observed in any of the samples providing confirmation that it is not formed in vivo. Linear regression analysis of the levels of dG-N^2-3-ABA and dG-C8-*N*-3-ABA in the rat DNA showed a reasonable correlation between the two methods ($R^2 = 0.88$ and 0.93, respectively). In summary, the mass spectrometric method is a faster, more automated analytical approach that also provides structural confirmation of the adducts detected by ^{32}P-postlabeling, and it has sufficient sensitivity and precision to analyze DNA adducts in animals exposed to 3-NBA or its hydroxylamine metabolite.

Introduction

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one[1], 3-NBA, **1**, Scheme 1) is a nitroaromatic ketone detected in the particulate matter of diesel engine exhaust and in urban air samples (*1*). 3-NBA (**1**) is one of the most potent mutagens in the Ames Salmonella assay (*1, 2*) and a potent mutagen (*3*) and a lung carcinogen in rats (*4*).

Scheme 1. Structures of 3-Nitrobenzanthrone (3-NBA, 1), 3-Aminobenzanthrone (3-ABA, 2), *N*-Hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA, 3), *N*-Acetoxy-3-aminobenzanthrone (*N*-AcO-3-ABA, 4), and *N*-Sulfoxy-3-aminobenzanthrone (*N*-SO$_4$-3-ABA, 5)



The worldwide use of diesel engines makes 3-NBA (**1**) a ubiquitous airborne contaminant, with levels up to 80 pg/m^3

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[1] Abbreviations: 3-nitro-7*H*-benz[*de*]anthracen-7-one (3-nitrobenzanthrone), 3-NBA; 3-amino-7*H*-benz[*de*]anthracen-7-one (3-aminobenzanthrone), 3-ABA; *N*-acetoxy-3-aminobenzanthrone, *N*-AcO-3-ABA; *N*-hydroxy-3-aminobenzanthrone, *N*-OH-3-ABA; *N*-sulfoxy-3-aminobenzanthrone, *N*-SO$_4$-3-ABA; 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone, dG-N^2-3-ABA; *N*-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone, dG-C8-*N*-3-ABA; 2-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone, dG-C8-C2-3-ABA; 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone, dA-N^6-ABA; selected reaction monitoring, (SRM); tetrahydrofuran, THF.

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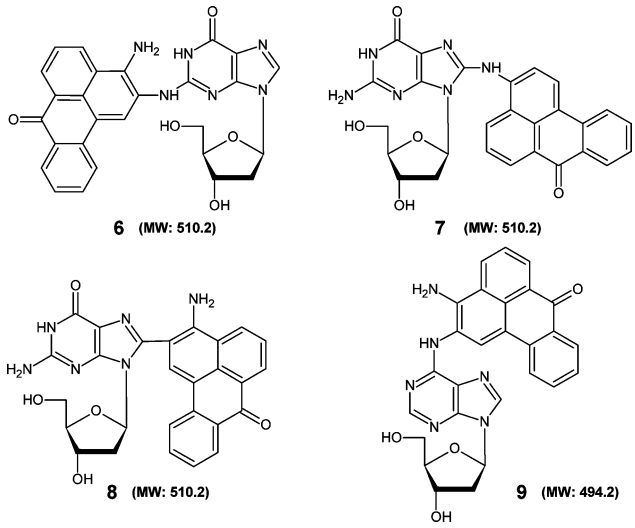
Scheme 2. Structures of the Main DNA Adducts Derived from 3-NBA in Vitro and in Vivo[a]



[a] 2-(2′-Deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA, **6**); *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA, **7**); 2-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone (dG-C8-C2-3-ABA, **8**); 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-3-ABA, **9**).

having been reported in workplace environments (*5*). Most probably as a result of the atmospheric washout of particulate matter, **1** has also been detected in rainwater (up to 2.6 ppb) (*6*) and soil samples (up to ca. 1.2 ppb) (*7, 8*), suggesting the possibility of human routes of exposure other than inhalation.

Although the induction of oxidative stress has been implicated in 3-NBA-induced genotoxicity (*9*−*12*), a large body of evidence demonstrates that this nitroketone is metabolically activated to electrophilic species capable of generating covalent DNA adducts [reviewed in ref *13*]. **1** is metabolized in vitro by mammalian cells to 3-aminobenzanthrone (3-ABA, **2**, Scheme 1) (*14*), a metabolite that has been detected in the urine of salt mine workers occupationally exposed to diesel engine emissions (*5*). A number of enzymatic in vitro bioassays conducted with human and rat hepatic cytosolic as well as microsomal preparations demonstrated that **1** can undergo enzymatic nitroreduction to its corresponding hydroxylamine (*N*-OH-3-ABA, **3**, Scheme 1) (*14*−*18*). This metabolite has also been shown to be formed by enzymatic *N*-oxidation of **2** (*19, 20*). Moreover, it has been demonstrated that *N*-OH-3-ABA (**3**) is a substrate for *N*-acetyltransferase (NAT) 1 and NAT2 as well as sulfotransferase (SULT) 1A1 and SULT1A2, and that the resulting esters (**4**, **5**, Scheme 1) react extensively with DNA to form covalent DNA adducts (*17, 21, 22*).

A number of covalent DNA adducts resulting from the exposure of experimental animals and human cells in culture to **1** or **2** have been detected by the ^{32}P-postlabeling method. In previous studies, the purine adducts 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA, **6**, Scheme 2); *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA, **7**, Scheme 2); and 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-3-ABA, **9**, Scheme 2) were identified as the predominant adducts formed (*23*−*25*). These studies have further indicated that 2-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone (dG-C8-C2-3-ABA, **8**, Scheme 2), an adduct readily formed in in vitro reactions of the model ester *N*-acetoxy-3-aminobenzanthrone (*N*-Aco-3-ABA, **4**, Scheme 1) with 2′-deoxyguanosine, does not seem to be formed in vivo.

A common feature of all the studies published to date on DNA adduct formation by **1** has been the use of the ^{32}P-postlabeling method for the detection and quantification of adducts. While this method has a very high sensitivity and requires low amounts of DNA (*26*), it can present a number of potential problems. Identification of the DNA adducts relies solely on cochromatography properties and quantification depends upon the enrichment procedure and enzymatic labeling efficiency, parameters that have been demonstrated to be structure-dependent (*26, 27*).

Among other methods for DNA adduct detection and quantification, mass spectrometry (MS) is an alternative or complementary approach to ^{32}P-postlabeling (*28*). MS methods present a number of advantages with respect to the identification and quantification of DNA adducts since they rely on the detection of adduct-specific ions and characteristic transitions of the ions being monitored, respectively, and the quantification relies upon the use of isotopically labeled internal standards.

In order to expand the range of available techniques for the detection and quantification of 2′-deoxyguanosine adducts formed by **1**, we have developed an assay using online column-switching HPLC coupled with electrospray tandem mass spectrometry incorporating isotopic dilution of internal standards.

Experimental Procedures

Caution. 1 and its derivatives are mutagens and potential human carcinogens and should be handled with care. Exposure to ^{32}P should be kept as low as possible, by working in a confined laboratory area, with protective clothing, Plexiglas shielding, Geiger counters, and body dosimeters. Waste materials must be discarded according to appropriate safety procedures.

Chemicals. All reagents, solvents, salmon testes DNA, and enzymes used in the hydrolysis of DNA to 2′-deoxynucleosides, mass spectrometry grade formic acid were purchased from Sigma Chemical Co. (Gillingham, UK) and were used as received. $^{15}N_5$-labeled 2′-deoxyguanosine was purchased from Spectra Stable Isotopes (Columbia, MD). The enzymes and chemicals used in the ^{32}P-postlabeling assay were obtained from commercial sources as described previously (*21, 26*). HPLC (fluorescence) grade methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). HPLC grade water, 18.2 MΩ cm output quality was obtained from Maxima Purification Equipment (Elga, High Wycombe, UK).

General Instrumentation. ^{1}H NMR spectra were acquired on a Bruker Avance 500 MHz with 5 mm BBO or QNP probe. Routine mass spectrometric analyses were conducted on a Micromass LCT mass spectrometer coupled to Waters Alliance 2795 HPLC system (Waters Corp., Milford, MA). The routine HPLC analyses and DNA adduct standard purifications were conducted on a Thermo Finnigan Surveyor HPLC (Thermo Finnigan Ltd., Hemel Hempstead, UK) equipped with a Luna C_{18} column, 5 μm, 250 × 4.6 mm, (Phenomenex, Waltham, MA). Routine HPLC analyses were conducted with a 20-min linear gradient of 10−100% acetonitrile/formic acid (99.9:0.1, v/v) and aqueous formic acid (0.1%, v/v), monitoring the eluate at a wavelength deemed suitable for the detection of each compound. The spectrophotometric analyses of DNA were carried out with a Beckman DU7 spectrophotometer (Beckman-Coulter, High Wycombe, Buckinghamshire, UK). Polyethyleneimine-cellulose (PEI-cellulose) thin-layer chromatography (TLC) plates used for ^{32}P-postlabeling were purchased from Macherey-Nagel (Düren, Germany) and were scanned using a Packard Instant Imager (Dowers Grove, IL).

Syntheses. 1 was synthesized by nitration of benzanthrone as described (*29*). **3** was prepared by palladium-catalyzed reduction of **1** with hydrazine as described previously (*30*), using tetrahydrofuran (THF) as the solvent. The identity of **1** and **3** was confirmed by high field ^{1}H NMR and electrospray mass spectrometry.

4 was prepared by selective *O*-acetylation of **3** with acetyl cyanide in anhydrous THF, essentially as reported (*23*). Given the expected instability of this ester, the solution thus obtained was

immediately used in reactions with 2′-deoxyguanosine or DNA without further characterization.

Preparation of 3-NBA-DNA Adduct Standards. The $^{15}N_5$-labeled and unlabeled DNA adduct standards were prepared by reacting freshly prepared solutions of **4** with $^{15}N_5$-labeled or unlabeled 2′-deoxyguanosine, respectively. In a typical reaction, 1.3 mg of **4** in 150 μL of THF was added to a solution of 1.3 mg of 2′-deoxyguanosine in 350 μL of water. Since some precipitation was observed upon addition of the acetoxyarylamine solution, 200 μL of dimethylformamide was added and the mixture was mixed thoroughly. The reaction mixture was incubated under agitation at 37 °C for 6 h and then taken to dryness under reduced pressure. The residue thus obtained was resuspended in 100 μL of methanol, and the insoluble fraction was discarded by centrifugation at ~13000*g* for 5 min. The methanolic solution containing the adducts was fractionated by reversed-phase HPLC with an 11-min linear gradient of 10 to 60% acetonitrile and 95% 50 mM ammonium acetate buffer, pH 5.7/acetonitrile (95:5, v/v). The eluate was monitored at 285 nm and the fractions corresponding to each dG-3-ABA adduct identified by comparison of the chromatogram with a chromatogram of a control experiment from which 2′-deoxyguanosine was excluded, and from the individual characteristic UV spectra of each adduct (*23*). Eluates were collected, pooled, and taken to dryness under reduced pressure. The adduct residues thus obtained were dissolved in methanol and the concentration of each standard solution was carefully determined by spectrophotometry in a quartz microcuvette using the extinction coefficients $\varepsilon_{267} = 26\,086\ M^{-1}\ cm^{-1}$ for dG-C8-*N*-3-ABA (**7**), $\varepsilon_{284} = 28\,370\ M^{-1}\ cm^{-1}$ for dG-N^2-3-ABA (**6**), and $\varepsilon_{284} = 36\,072\ M^{-1}\ cm^{-1}$ for dG-C8-C2-3-ABA (**8**). The methanolic stock solutions were kept at −20 °C in tightly sealed glass vials with Teflon-lined septa to minimize solvent evaporation.

In Vitro N-AcO-3-ABA-modified DNA Samples. A range of DNA samples with different levels of in vitro modification were prepared by adding 1 mL of seriated dilutions (1:10) of **4** in THF to 5 mL of solutions of salmon testes DNA (1 mg/mL in 25 mM phosphate buffer, pH 7.0). The estimated concentrations of the acetoxyarylamine in the organic solution ranged from 0.5 mg/mL to 5×10^{-10} mg/mL. DNA samples were incubated overnight at 37 °C and washed with 1 vol of ethyl acetate, 1 vol of water-saturated *n*-butanol, and the DNA was precipitated by addition of 0.1 vol of 5 M NaCl and 2 vol of ice-cold ethanol. DNA samples were centrifuged, the pellet thus obtained was washed twice with 5 mL of 70% ice-cold ethanol/water and the residue finally obtained upon centrifugation was redisolved in 4 mL of 5 mM BisTris buffer, pH 7.1, 0.1 mM EDTA, allowing an overnight rehydration at 4 °C before freezing the samples at −80 °C until further use. The DNA concentration in the final samples was quantified by UV spectrophotometry.

Enzymatic Hydrolysis of in Vitro Modified DNA for HPLC-ESI-MS/MS Analysis. Each of the in vitro modified DNA samples (1.0 mg) was dried down using a centrifugal vacuum evaporator. The dried DNA samples were dissolved in 990 μL of 50 mM BisTris, 0.1 mM EDTA, pH 7.1 buffer plus 10 μL of 1 M $MgCl_2$ and incubated with 100 μL of deoxyribonuclease I (2 mg/mL dissolved in 0.15 M NaCl, 10 mM $MgCl_2$) at 37 °C for 6 h. The samples were further incubated with 60 μL of snake venom phosphodiesterase I from *Crotalus adamanteus* (0.001 U/μL dissolved in 0.11 M Tris-HCl, 0.11 M NaCl, 15 mM $MgCl_2$, pH 8.9) and 26.2 μL of alkaline phosphatase from *Escherichia coli* (0.305 U/μL) at 37 °C for 15 h. The hydrolyzed DNA samples were centrifuged at 14 000 rpm for 2 min and stored at −80 °C. Aliquots of the hydrolyzed DNA samples were removed to which were added 1.33 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards (100 fmol/μL) and dried down using a centrifugal vacuum evaporator. The dried samples were dissolved in 20 μL of HPLC grade water/methanol (80:20, v/v) and transferred to HPLC vials with low volume inserts. A 15 μL aliquot of each sample was injected on to the online column switching HPLC-ESI-MS/MS, equivalent to 5, 20, 50, or 100 μg of hydrolyzed DNA depending on the extent of modification by **4** and 1.0 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards on column.

Treatment of Animals. Female Sprague-Dawley rats, (~360 g; 3/group) were treated with 0.2 or 2 mg/kg body weight (bw) of **1** by intratracheal instillation under ether anesthesia (*31, 32*). **1** was dissolved in tricaprylin at a concentration of 0.4 and 4 mg/mL for the low and high dose group, respectively, resulting in a volume of 180 μL administered per rat. **1** was administered to rats at the bronchial bifurcation by injection through a tracheal cannula. Control rats were treated with vehicle only (180 μL of tricaprylin). The animals were killed after 48 h, organs (lung, liver, kidney, pancreas, and small intestine) were collected, snap-frozen in liquid nitrogen and stored at −80 °C until DNA isolation by standard phenol/chloroform extraction.

Female Wistar rats (~250 g; $n = 3$) were treated with 10 mg/kg bw of **3** by intraperitoneal injection (*33*). **3** was dissolved in tricaprylin at a concentration of 5 mg/mL, resulting in a volume of 500 μL administered per rat. Control rats were treated with vehicle only (500 μL tricaprylin). Animals were killed 24 h after treatment and the organs (lung, liver, kidney, spleen, pancreas and colon) collected, immediately frozen in liquid nitrogen and stored at −80 °C until DNA isolation by standard phenol/chloroform extraction.

All animal experiments were carried out under license in accordance with the law, and following local ethical review.

Enzymatic Hydrolysis of Animal DNA for HPLC-ESI-MS/MS Analysis. Rat DNA samples (66.7 μg) were dried down using a centrifugal vacuum evaporator. The dried DNA samples were dissolved in 99 μL of 50 mM BisTris, 0.1 mM EDTA, pH 7.1 buffer plus 1.0 μL of 1 M $MgCl_2$ and incubated with 10 μL of deoxyribonuclease I (2 mg/mL dissolved in 0.15 M NaCl, 10 mM $MgCl_2$) at 37 °C for 6 h. The samples were further incubated with 6.0 μL of snake venom phosphodiesterase I from *C. adamanteus* (0.001 U/μL dissolved in 0.11 M Tris-HCl, 0.11 M NaCl, 15 mM $MgCl_2$, pH 8.9) and 3.28 μL of alkaline phosphatase from *E. coli* (0.305 U/μL) at 37 °C for 15 h. The hydrolyzed DNA samples were centrifuged at 14,000 rpm for 2 min and the supernatant transferred to a new eppendorf tube to which was added 1.33 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards (100 fmol/ μL) and then dried down using a centrifugal vacuum evaporator. The dried samples were dissolved in 20 μL of HPLC grade water/methanol (80:20, v/v) and transferred to HPLC vials with low volume inserts. A 15 μL aliquot of each sample was injected on to the online column switching HPLC-ESI-MS/MS equivalent to 50 μg of hydrolyzed DNA and 1.0 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards on column.

Online Column-Switching HPLC-ESI-MS/MS Analysis. The online column switching valve system consisted of an automated switching valve (motorized two-position six-port valve, Waters Ltd., Hertfordshire, UK) connected to pump A incorporating an autosampler (Waters Alliance 2695 separations module with a 100 μL injection loop) and an isocratic pump B (Gynotek GmbH, Germering, Germany). Pump A was connected via the switching valve to the trap column, Synergi Fusion-RP 80A C_{18}, 4 μm, 30 × 2.0 mm column attached to a KrudKatcher disposable precolumn (0.5 μm) filter (Phenomenex, Macclesfield, UK) and Pump B was connected via the switching valve to the analytical column, Synergi Fusion-RP 80A C_{18} (4 μm, 250 × 2.0 mm) column attached to a Synergi Fusion-RP 80A C_{18} (4 μm, 4.0 × 2.0 mm) guard column and KrudKatcher disposable precolumn (0.5 μm) filter. The outlet of the analytical column was directly connected to the mass spectrometer. The configuration of the online column switching valve system is summarized in the Supporting Information.

Sample Loading. A 15-μL aliquot of the in vitro modified (equivalent to 5, 20, 50, or 100 μg of hydrolyzed DNA) or animal (equivalent to 50 μg of hydrolyzed DNA) DNA sample containing 1 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards was injected onto the trap column using pump A with the switching valve in position 1. The impurities on the trap column were eluted to waste using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120

μL/min. The following gradient was used: 0 min-15%B, 10 min-15%B, 20 min-35%B, 30 min-35%B, 30.1 min-100%B (200 μL/min), 35 min-100%B (200 μL/min), 35.1 min-15%B and 45 min-15%B. Concurrently isocratic flow at 120 μL/min via the analytical column, which was eluted with 0.1% formic acid/acetonitrile (67:33, v/v), was maintained to the mass spectrometer by means of pump B.

Sample Elution. At 19.0 min the switching valve was switched to position 2 allowing the purified dG-3-ABA adducts to be back-flushed from the trap column onto the analytical column and then subsequent elution into the mass spectrometer. The isocratic flow was maintained by pump B at flow rate of 120 μL/min with 0.1% formic acid/acetonitrile (67:33, v/v) for 11.0 min. Concurrently the flow from pump A bypassed the trap column and was diverted directly to waste. At 30.0 min the switching valve was switched back to position 1 and the configuration of the online column switching system reverted back to initial conditions as described for the sample loading above. The total run time was 45 min.

Mass Spectrometry. A Waters Micromass Quattro Ultima Pt (Micromass, Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface was used. The temperature of the electrospray source was maintained at 110 °C and the desolvation temperature at 350 °C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas (25 L/h). The capillary voltage was set at 3.20 V. The cone and RF1 lens voltages were 42 and 25 V, respectively. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The dwell time was set to 200 ms and the resolution was two *m*/*z* units at peak base. The mass spectrometer was tuned by using a standard solution of 2′-deoxyguanosine (10 pmol/μL) in 0.1% formic acid/acetonitrile (67:33, v/v) introduced by continuous infusion at a flow rate of 10 μL/min with a Harvard model 22 syringe pump (Havard Apparatus Ltd., Edenbridge, UK). The hydrolyzed DNA samples were analyzed in positive ESI MS/MS selected reaction monitoring (SRM) mode for the $[M + H]^+$ ion to base $[B + H_2]^+$ transitions of *m*/*z* 511 to 395 for **6**, **7**, and **8** and *m*/*z* 516 to 400 for [$^{15}N_5$]dG-C8-*N*-3-ABA, [$^{15}N_5$]dG-N^2-3-ABA plus [$^{15}N_5$]dG-C8-C2-3-ABA. The level of each adduct in the DNA samples was determined from the ratio of peak areas of the respective [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards.

Calibration Lines for dG-3-ABA Adducts with DNA Matrix. The calibration lines were constructed by the addition of different amounts of the unlabeled dG-3-ABA standards (ranging from 2.0 to 4000 fmol) plus 1.0 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards to 50 μg of enzymatically hydrolyzed calf thymus DNA as the matrix. The standards were subjected to the online column switching HPLC-ESI-MS/MS analysis procedure as described above.

DNA Adduct Analysis by ^{32}P-Postlabeling. DNA adducts were measured for each DNA sample using thin-layer chromatography (TLC) ^{32}P-postlabeling and the butanol extraction enrichment version of the assay essentially as described previously (*21, 26*). The chromatographic conditions for TLC on polyethyleneimine-cellulose (PEI-cellulose) were (*16, 21*): D1, 1.0 M sodium phosphate, pH 6; D3, 4 M lithium-formate, 7 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8. After chromatography TLC sheets were scanned and DNA adduct levels (RAL, relative adduct labeling) were calculated from adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. 3-NBA-derived DNA adducts were identified using authentic standards as described previously (*23*). Results were expressed as DNA adducts/10^8 nucleotides.

Results

Preparation of Labeled and Unlabeled 3-NBA-DNA Adduct Standards. Accurate DNA adduct quantitation by mass spectrometry is best achieved by coupling selected reaction monitoring (SRM) with the use of stable isotope-labeled internal standards. In order to prepare labeled and unlabeled standards of the major 3-NBA adducts with 2′-deoxyguanosine, **4**, prepared by selective *O*-acetylation of the respective hydroxylamine, was reacted with 2′-deoxyguanosine or $^{15}N_5$-labeled 2′-deoxyguanosine. The fractionation of the reaction products by reversed-phase HPLC conveniently afforded the desired labeled and unlabeled standards of **6**, **7**, and **8**. The identity of each adduct was determined by comparison of its UV spectrum with published spectra (*23*). The electrospray mass spectral analysis of the DNA adduct standards was entirely consistent with the expected structures, with protonated molecular ions at *m*/*z* 511 and *m*/*z* 516 and protonated adducted bases at *m*/z 395 and *m*/*z* 400, respectively, for the unlabeled and $^{15}N_5$-labeled adducts. Standard methanolic solutions of each unlabeled and labeled adduct were prepared using the corresponding extinction coefficients. These extinction coefficients were determined experimentally by carefully weighing **6** and **7** on a precision microbalance or by ^{1}H NMR using *t*-butanol as an internal standard for **8** (data not shown). The labeled adduct standards were chemically pure, as determined both by HPLC-UV, and by full scan HPLC-ESI-MS/MS. No unlabeled adduct was detectable in the isotopic standard solutions as determined by HPLC-ESI-MS/MS operated in SRM mode.

In Vitro Modification of Salmon Testis DNA with N-AcO-3-ABA. 4 was prepared by selective *O*-acetylation of **3** and the ester was reacted with salmon testis DNA at different ratios, varying by 10-fold increments from 0.5×10^{-10} to 0.5 mg of acetoxylamine/mg of DNA. After the DNA was purified by solvent extractions and precipitation, aliquots of the highest modified samples, containing 100 μg of DNA, were hydrolyzed enzymatically to deoxynucleosides (*34*) and the hydrolysates were analyzed directly by HPLC with UV detection. **6** and **7** were readily identified based upon their characteristic UV spectra and cochromatography with authentic standards prepared as outlined above. These adducts were also detectable in the UV traces of the hydrolysates of the DNA modified at the ratios of 0.05 and 0.005 mg of acetoxylamine/mg of DNA, albeit close to the limit of detection for **7** in the lowest modified samples. The analysis of the UV traces failed to demonstrate the formation of **8**.

Online Column Switching HPLC-ESI-MS/MS Analysis. Mass spectrometric analysis of the three dG-3-ABA adduct standards was performed in positive ESI MS/MS mode and resulted in the typical product ion spectra that are observed for adducted 2′-deoxynucleosides. The collision induced dissociation product ion mass spectrum consisted of the adducted base as being the most abundant product ion formed from the $[M + H]^+$ ion at *m*/*z* 511. The adducted base product ion $[B + H_2]^+$ at *m*/*z* 395 was formed by the cleavage of the glycosidic bond and the accompanied proton transfer from the 2′-deoxyribose moiety to the adducted base. Similar fragmentation patterns with the *m*/*z* increased by 5 were observed for the $[M + H]^+$ ions for the corresponding [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards.

Initial experiments involved optimization of the mobile phase conditions for the separation of dG-3-ABA adducts on the trap column. A gradient mobile phase system consisting of 0.1% formic acid and acetonitrile was found to give the optimal separation on the trap column with retention times of 21.30 min for **7**, 21.83 min for **6**, and 23.72 min for **8**. The online column switching valve system was configured as shown in the Supporting Information and the switching valve was switched to position 2 at 19.0 min following injection of the hydrolyzed DNA sample. This allowed for unmodified 2′-deoxynucleosides plus any other matrix impurities that may have been present to

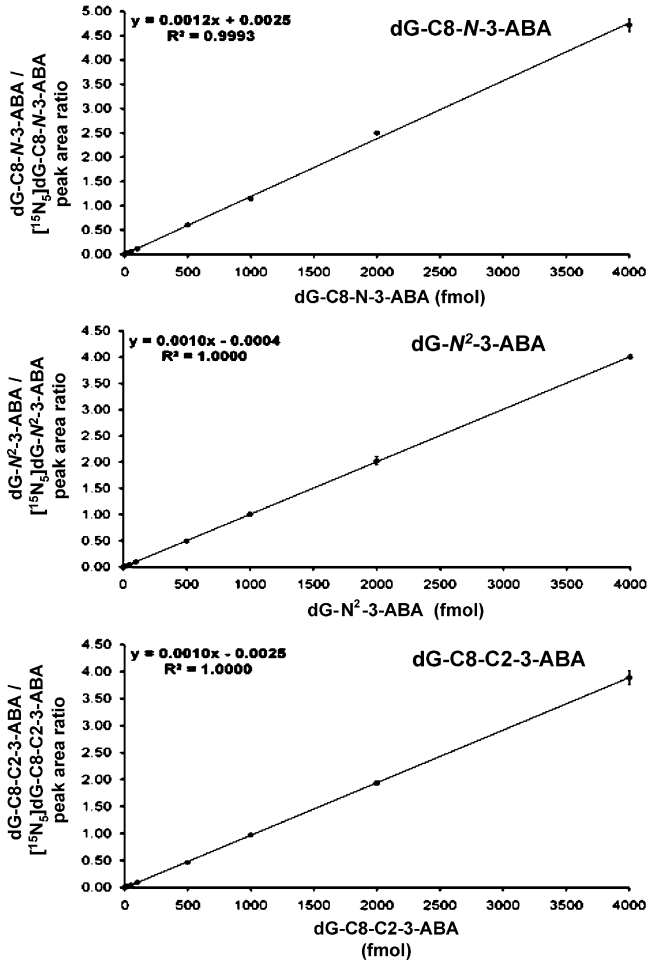


Figure 1. Isotope dilution calibration curves for dG-C8-*N*-3-ABA, dG-N^2-3-ABA, and dG-C8-C2-3-ABA.

eluted to waste while the three dG-3-ABA adducts were back-flushed onto the analytical column for analysis by mass spectrometry.

Calibration curves were constructed by preparing a series of standards each containing varying amounts of dG-3-ABA adducts and 1.0 pmol of each of the [$^{15}N_5$]-labeled dG-3-ABA stable isotope internal standards added to 50 μg of enzymatically hydrolyzed calf thymus DNA to account for any matrix effects. The response was linear over a range of 2.0 up to 4000 fmol for all three dG-3-ABA adducts (Figure 1), a range corresponding to approximately 1.2−2500 adducts in 10^8 deoxynucleosides.

The approximate limits of detection (LOD, S/$N \geq 3$) and quantification (LOQ, S/$N \geq 10$), determined by spiking known amounts of each adduct and respective labeled internal standard in calf thymus DNA enzymatic hydrolyzate, were 2 and 10 fmol, respectively, on-column.

The HPLC-ESI-MS/MS method precision was determined by analyzing on three consecutive days, aliquots ($n = 3-5$) of DNA modified in vitro with **4**. Excluding the samples whose modification levels approached the limit of quantification of the technique, the intra- and interday variations observed were typically less than 7% RSD for **7** and **6**, and less than 12% for **8** (see Supporting Information). The average accuracy of the method at the LOQ, determined by spiking known amounts of each adduct and respective labeled internal standard in calf thymus DNA enzymatic hydrolyzate was 108% for dG-C8-*N*-3-ABA, 89% for dG-N^2-3-ABA, and 99% for dG-C8-C2-3-ABA.

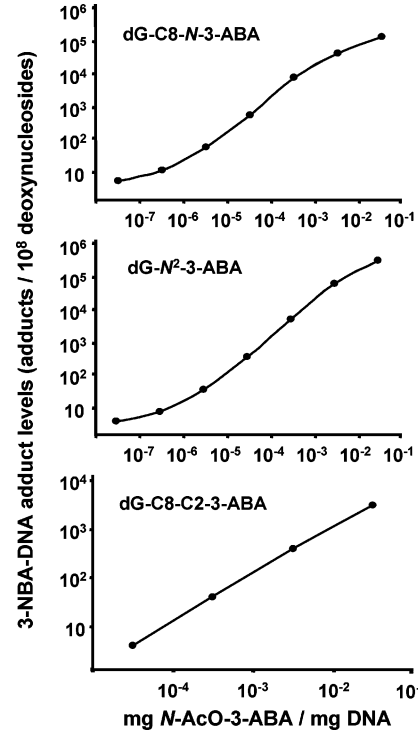


Figure 2. Levels of dG-C8-*N*-3-ABA, dG-N^2-3-ABA, and dG-C8-C2-3-ABA detected in salmon testis DNA modified in vitro with varying ratios of *N*-AcO-3-ABA as determined by online column-switching HPLC-ESI-MS/MS. The adduct levels are expressed as the number of adducts per 10^8 unmodified deoxynucleosides.

A reasonable linearity was observed in function of the DNA modification level (mg of **4**/mg of DNA) for the three adducts (Figure 2). **7** and **6** were detected at comparable levels, while **8** consistently represented less than 1% of the overall adduct content (Figure 2). No observable responses for **6**, **7**, or **8** were detected in control DNA in the absence of added adduct standards.

HPLC-ESI-MS/MS and ^{32}P-Postlabeling Analyses of DNA Samples Modified in Vivo with 3-NBA or N-OH-3-ABA. Female Sprague−Dawley rats were treated by intratracheal instillation with a single dose of 0.2 or 2 mg/kg bw of 3-NBA (**1**). Female Wistar rats were treated by intraperitoneal injection with a single dose of 10 mg/kg bw of *N*-OH-3-ABA (**3**). In both cases, control animals were treated with the vehicle alone. DNA isolated from lung, liver, kidney, spleen, pancreas, and colon was analyzed by HPLC-ESI-MS/MS and ^{32}P-postlabeling. Figure 3 depicts representative SRM ion chromatograms obtained for the online column-switching HPLC-ESI-MS/MS analysis of lung DNA from rats treated with 10 mg/kg bw of **3** (panel A) or 0.2 mg/kg bw of **1** (panel B). In both treatments the detection of the **6** and **7** was easily accomplished by comparison of the coeluting peaks in the SRM transition traces corresponding to the adducts (*m/z* 511 > 395) and respective internal standards (*m/z* 516 > 400). Conversely, while the [$^{15}N_5$]dG-C8-C2-3-ABA internal standard was apparent in the *m/z* 516 > 400 transition trace at *ca.* 33 min, the *m/z* 511 > 395 transition trace failed to show evidence of formation of **8** in all samples analyzed. No observable responses for **6**, **7**, or **8** were detectable in the DNA obtained from the control animals treated with the vehicle alone (data not shown). As previously observed in the analysis of the in vitro modified DNA samples, repeat analyses of the rat DNA samples by HPLC-ESI-MS/MS revealed good precision, with an average quantitation variability of less than 7% (data not shown).

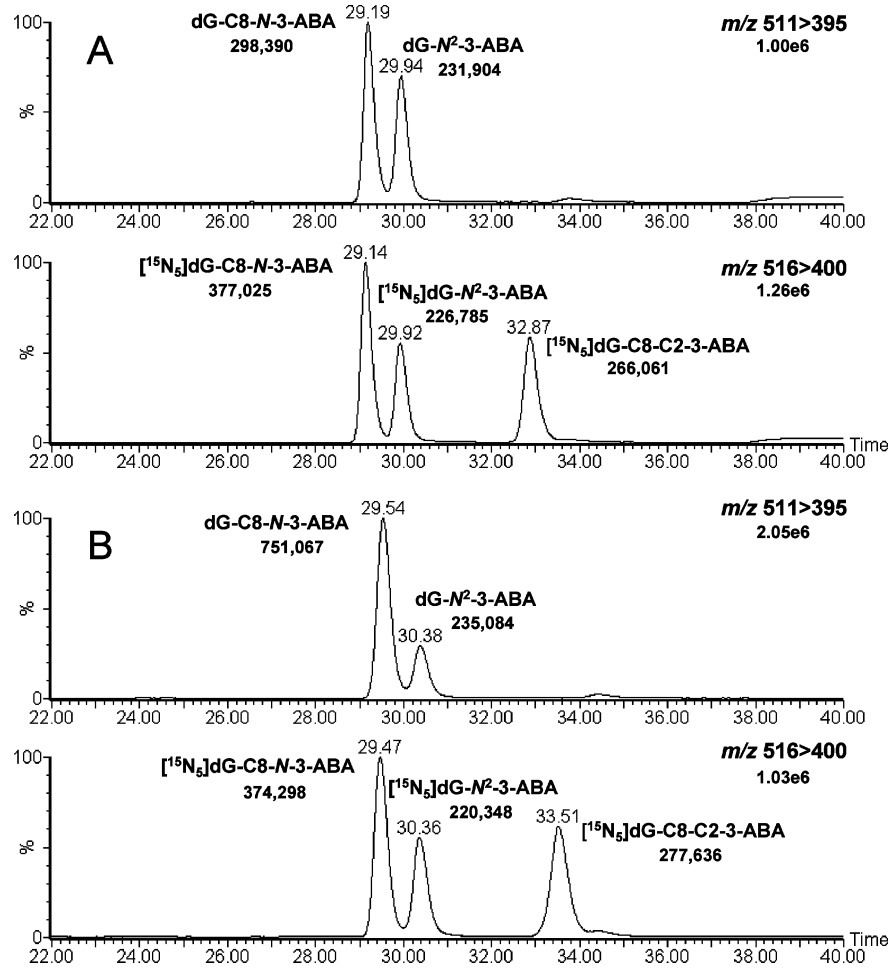


Figure 3. Representative online column-switching HPLC-ESI-MS/MS SRM ion chromatograms for the determination of 3-NBA-DNA adducts in lung DNA from rats treated with 10 mg/kg bw of *N*-OH-3-ABA (panel A) or 0.2 mg/kg bw of 3-NBA (panel B). The animals were treated by intratracheal instillation (3-NBA) or by intraperitoneal injection (*N*-OH-3-ABA) as outlined in Experimental Procedures. The ion chromatograms depict signal intensity versus time for the SRM transitions corresponding to the loss of deoxyribose from the protonated adducts (*m*/*z* 511 > 395) and respective $^{15}N_5$-labeled internal standards (*m*/*z* 516 > 400; 1.0 pmol each). The integrations are specified for each adduct peak. The elution conditions are outlined in Experimental Procedures.

Figure 4 depicts a comparison of the levels of **7** and **6** in selected tissues of rats treated by intratracheal instillation with 0.2 mg/kg bw 3-NBA (panels A and B), 2 mg/kg bw 3-NBA (panels C and D) or by intraperitoneal injection with 10 mg/kg bw **3** (panels E and F), as determined by HPLC-ESI-MS/MS (gray bars) or ^{32}P-postlabeling (black bars). The error bars represent the standard error of the mean for the determinations of each adduct in organs from three animals exposed to each treatment. Although a dose-response was observed in the animals treated with **1**, both as determined by HPLC-ESI-MS/MS or ^{32}P-postlabeling, an overall tendency for lower average quantification results was observed using the ^{32}P-postlabeling methodology. This trend was more pronounced in the samples obtained from animals exposed to **3**. Figure 5 depicts a linear regression analysis of the levels of **7** (panel A) and **6** (panel B) in DNA samples from lung, kidney, liver, colon, and pancreas analyzed in this study by HPLC-ESI-MS/MS and ^{32}P-postlabeling. Although a reasonable correlation was observed between the two methodologies, the slope of the curves obtained for each adduct (0.71 for **7**, and 0.38 for **6**) indicates a tendency of the ^{32}P-postlabeling method to underestimate DNA adduct levels in comparison with the mass spectral methodology.

Discussion

Mass spectrometry is a technique presenting great potential when applied to the detection and quantification of DNA adducts due to its very high sensitivity and chemical specificity, which allows the identification of specific DNA adducts even in a complex matrix of structurally related DNA adducts (*28*). We have previously successfully applied this methodology, using isotopically labeled internal standards, to the detection and quantification of a varied range of DNA adducts, including adducts of tamoxifen, benzo[*a*]pyrene, glycidamide, and 8-oxo derivatives of purines (*34*−*37*).

In the present study, we have explored the HPLC-ESI-MS/MS methodology as a suitable routine analytical methodology for the detection and quantification of 3-NBA-derived DNA adducts formed in vivo. A method was developed based on online column-switching and SRM of the transitions from the protonated deoxynucleoside molecule to the positively charged purine base of the adducts **6**, **7**, and **8**, and respective $^{15}N_5$-labeled internal standards. The method, validated within a range of 1.2−2500 adducts per 10^8 deoxynucleosides, presented good statistical performance (RSD < 12%) and estimated LOD and LOQ of 2 and 10 fmol, respectively, on column for all adducts. These values correspond to an LOD of approximately 1.2 adducts in 10^8 deoxynucleosides and an LOQ of approximately 6 adducts in 10^8 deoxynucleosides in a 50 μg of DNA sample.

In order to assess the performance of the HPLC-ESI-MS/MS method for the detection and quantification of 3-NBA-derived DNA adducts formed in vivo, DNA samples obtained from different organs of rats treated either by intratracheal

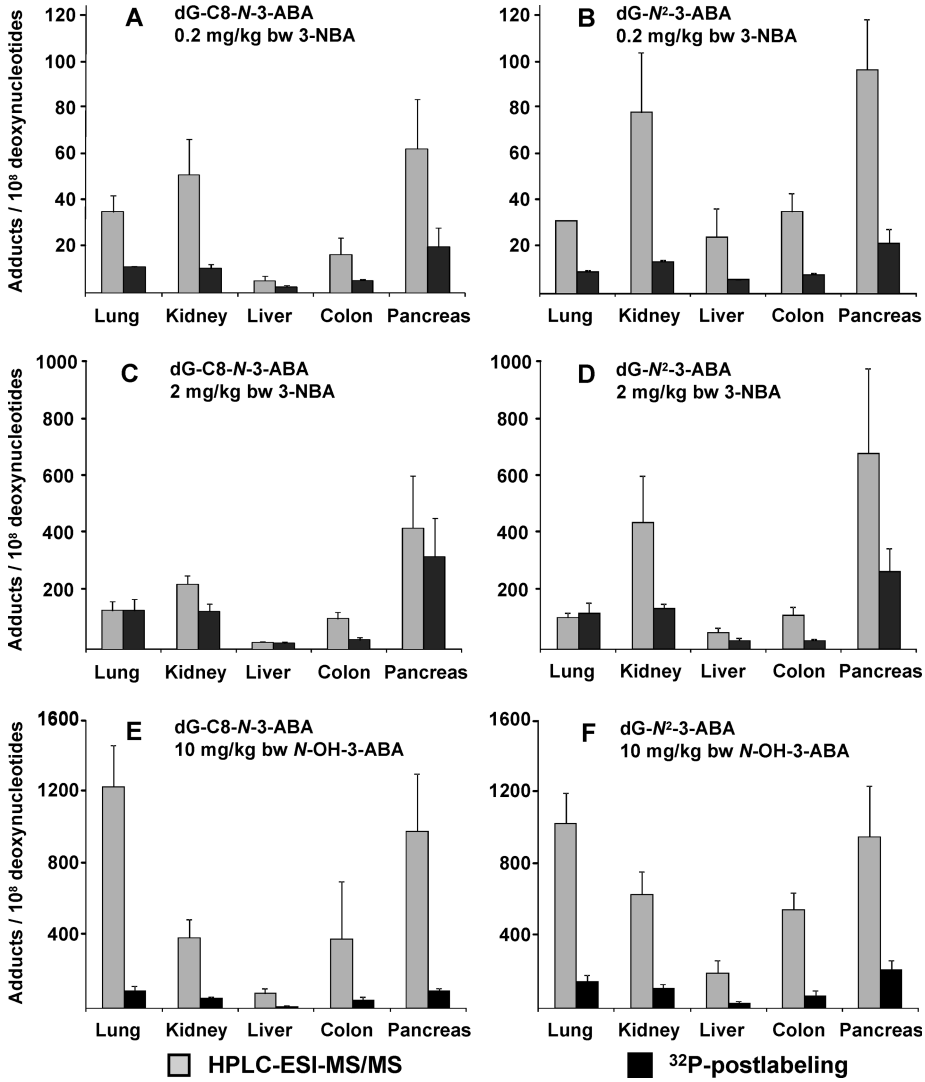


Figure 4. Comparison of the levels of the covalent adducts dG-C8-*N*-3-ABA and dG-N^2-3-ABA in lung, kidney, liver, colon, and pancreas of rats treated by intratracheal instillation with 0.2 mg/kg bw 3-NBA (panels A and B), 2 mg/kg bw 3-NBA (panels C and D) or by intraperitoneal injection with 10 mg/kg bw *N*-OH-3-ABA (panels E and F), as determined by HPLC-ESI-MS/MS (gray bars) or ^{32}P-postlabeling (black bars). The DNA adduct levels are expressed in adducts per 10^8 deoxynucleosides (HPLC-ESI-MS/MS) or 2′-deoxynucleotides (^{32}P-postlabeling) and the error bars represent the standard error of the mean ($n = 3$). The animal treatment conditions and analytical procedures are detailed in Experimental Procedures.

instillation with **1** or by intraperitoneal injection with **3** were analyzed by the mass spectral and ^{32}P-postlabeling methods. The two methodologies revealed very similar results from a qualitative standpoint, with **6** and **7** being detected in all samples, in contrast to **8**, which was not detected in any of the samples. The absence of this adduct in the rodent samples is consistent with the published literature (*23, 30*), and with observations in the present study, where it was detected at much lower levels than the other adducts in in vitro modified DNA. Interestingly, the formation of **8** proceeded with good yields in the syntheses conducted with isolated deoxynucleosides, suggesting that the conformation of the DNA plays an important role in the regioselectivity of the adduction reaction of 2′-deoxyguanosine. It is noteworthy that although a number of 3-NBA-DNA adducts formed with deoxyadenosine have also been reported (*3, 16, 23, 30, 38*), mutational spectrum analysis indicates that GC → TA transversions constitute the predominant mutations in tissues of animals exposed to **1**, suggesting that the 2′-deoxyguanosine adducts play a pivotal role in tumor development (*39*−*41*).

From a quantitative standpoint, both methodologies revealed in general comparable levels of **6** and **7** in the tissues of animals treated intratracheally with **1**, and a clear dose-response was observed between the 0.2 and 2 mg/kg bw dose groups. Approximately equal levels of the two adducts were also detected in the animals treated i.p. with **3**. It should be noted that the variability shown by the error bars represents mainly biological variability and not analytical imprecision, as the mean coefficient of variation of the mass spectral determinations was less than 7% in the duplicate or triplicate analyses conducted on each sample. Regression analysis of the levels of **6** and **7** in all the rodent organs analyzed in this study indicated a reasonable correlation between the two methodologies ($R^2 \approx 0.9$ for both adducts). However, the slope of the regression lines indicates an overall tendency of the ^{32}P-postlabeling method to give a lower estimate of the DNA adduct levels in comparison with the mass spectral method. This underestimation, more pronounced for **6** (slope ≈ 0.4) than for **7** (slope ≈ 0.7), may reflect the low recoveries reported for 3-NBA-DNA adducts under ^{32}P-postlabeling conditions (*30*), and is consistent with previous observations in the analysis of DNA adducts derived from tamoxifen (*42*) or benzo[*a*]pyrene (*36*).

In conclusion, we have developed a methodology based on online column-switching HPLC coupled with tandem mass spectrometry with sufficient sensitivity and precision to be useful

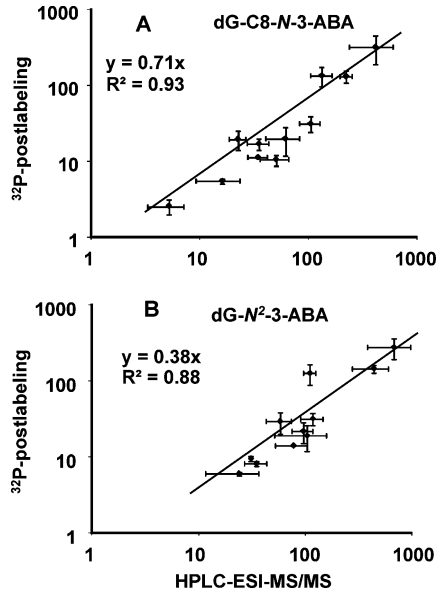


Figure 5. Comparison of the levels of the covalent adducts dG-C8-*N*-3-ABA (panel A) and dG-N^2-3-ABA (panel B) in lung, kidney, liver, colon, and pancreas of rats treated by intratracheal instillation with 0.2 mg/kg bw 3-NBA, 2 mg/kg bw 3-NBA, or by intraperitoneal injection with 10 mg/kg bw *N*-OH-3-ABA as determined by HPLC-ESI-MS/MS or ^{32}P-postlabeling. The DNA adduct levels are expressed in adducts/10^8 deoxynucleosides (HPLC-ESI-MS/MS) or 2′-deoxynucleotides (^{32}P-postlabeling) and the error bars represent the standard error of the mean ($n = 3$). The animal treatment conditions and analytical procedures are detailed in Experimental Procedures.

in the analysis of 3-NBA-DNA adducts formed in in vitro and in vivo experiments. While this mass spectral method has higher sample requirements and is generally less sensitive than the ^{32}P-postlabeling method, it presents the clear advantages of providing information on the chemical identity of the adducts and constituting a more automated and faster analytical method. We are currently investigating the possible use of nanoflow chromatography coupled with nanoelectrospray-tandem mass spectrometry (*43, 44*) as a means to bridging the current gap in sample requirements and sensitivity between the two methodologies. In the long term, these approaches aim to improve and validate methods for biomonitoring human exposure to genotoxic carcinogens.

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Supporting Information Available: Configuration of the column switching valve system used for online pre-purification of the DNA hydrolysate samples; table of assessment of the precision of the HPLC-ESI-MS/MS method for the quantification of 3-NBA-DNA adducts. This material is available free of charge via the Internet at http://pubs.acs.org.

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