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Ultra-sensitive High Resolution Mass Spectrometric Analysis of a DNA Adduct of the Carcinogen Benzo[*a*]pyrene in Human Lung

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Abstract

Benzo[*a*]pyrene (BaP), an archetypical polycyclic aromatic hydrocarbon, is classified as "carcinogenic to humans" and is ubiquitous in the environment, as evident by the measurable levels of BaP metabolites in virtually all human urine samples examined. BaP carcinogenicity is believed to occur mainly through its covalent modification of DNA, resulting in the formation of BPDE-N^2-dG, an adduct formed between deoxyguanosine and a diol epoxide metabolite of BaP, with subsequent mutation of critical growth control genes. In spite of the LC-MS based detection of BPDE-N^2-dG in BaP-treated rodents, and indirectly through HPLC-fluorescence detection of BaP-7,8,9,10-tetraols released from human DNA upon acid hydrolysis, BPDE-N^2-dG adducts have rarely if ever been observed directly in human samples using LC-MS techniques, even though sophisticated methodologies have been employed which should have had sufficient sensitivity. With this in mind, we developed an LC-ESI-MS/MS methodology employing high resolution/accurate mass analysis for detecting ultra-trace levels of these adducts. These efforts are directly translatable to the development of sensitive detection of other small molecules using trap-based LC-ESI-MS/MS detection. The developed methodology had an LOD of 1 amol of BPDE-N^2-dG on-column, corresponding to 1 BPDE-N^2-dG adduct/10^{11} nucleotides (1 adduct/10 human lung cells) using 40 μg of human lung DNA. To our knowledge, this is the most sensitive DNA adduct quantitation method yet reported, exceeding the sensitivity of the ^{32}P-postlabeling assay (~1 adduct/10^{10} nucleotides). 29 human lung DNA samples resulted in 20 positive measurements above the LOD, with smoker and non-smoker DNA containing 3.1 and 1.3 BPDE-N^2-dG adducts/10^{11} nucleotides, respectively.

Graphical abstract

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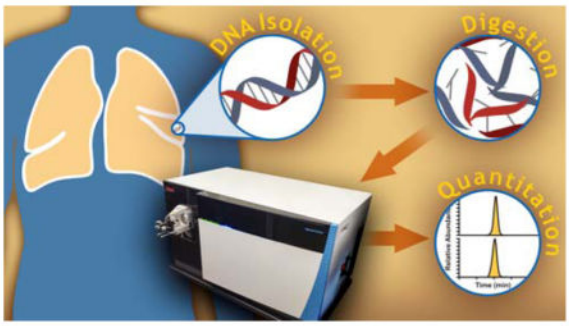
Supporting Information

Supporting Information Available:

Discussion of DNA hydrolysis conditions, injection times, and resolution; chemical structures and fragmentation spectra; plots of quadrupole ion transmission efficiency and measured spiked BPDE-N^2-dG; summary of measured BPDE-N^2-dG levels in human lung samples.



Benzo[*a*]pyrene (BaP, **1**, Scheme 1) is perhaps the most intensely studied of all chemical carcinogens.[1] It is the archetype of the broad class of carcinogenic combustion products – the polycyclic aromatic hydrocarbons (PAH) – ubiquitous environmental compounds formed in the incomplete combustion of organic matter. Exposure to PAH is virtually unavoidable due to their presence in the human diet, polluted air, cigarette smoke, and other environments where combustion occurs. Indeed, the presence of PAH metabolites in virtually all human urine samples has been confirmed in the U.S. National Health and Nutrition Examination Study and related large studies.[2–4] The research focus on BaP among various PAH is due to its powerful carcinogenicity in laboratory animals. This property, together with appropriate mechanistic data, led to its classification as "carcinogenic to humans" by the International Agency for Research on Cancer.[5]

BaP requires metabolism to modify the DNA of critical growth control genes and thus exert its carcinogenic effects. The DNA binding pathway supported by the most evidence including the genotoxicity of metabolites in *in vitro* systems, metabolism and DNA binding studies in laboratory animals and human tissues, and the carcinogenicity of its metabolites, is illustrated in Scheme 1.[5–12] The initial step is catalyzed by cytochrome P450s 1A1 and 1B1 resulting in the formation of BaP-7,8-epoxide (**2**). This epoxide is converted to BaP-7,8-diol (**3**) with catalysis by epoxide hydrolase (EH). Diol **3** is then further oxidized to produce BaP-7,8-diol-9,10-epoxide (**4**) with predominantly 7*R*,8*S*,9*S*,10*R* absolute configuration as illustrated in Scheme 1. This diol epoxide reacts easily with DNA to produce well characterized adducts among which BPDE-N^2-dG (**5**) predominates. Studies of mutations in the *p53* tumor suppressor gene and mutational signatures associated with cigarette smoking are also consistent with this pathway.[13,14] While other mechanisms of metabolic activation of BaP have been proposed,[5,15–17] the preponderance of evidence favors the diol epoxide pathway.

Therefore, based on this massive amount of unambiguous supporting data, one would expect to find BPDE-N^2-dG (**5**) in DNA isolated from tissues or cells of people exposed to BaP since this adduct has been routinely detected in DNA of laboratory animals dosed with BaP. While multiple studies have used HPLC-fluorescence and related techniques to analyze BaP-7,8,9,10-tetraols released from human DNA upon acid hydrolysis, relatively few have used mass spectrometry to quantify BPDE-N^2-dG itself. We are aware of only 5 such studies in the literature. Two of them examined human lung tissue; one did not detect BPDE-N^2-dG in any of ten samples analyzed (detection limit, 1–3 adducts per 10^8 nucleotides)[18] while the

second proved negative in 25 of 26 samples (detection limit, 0.3 adducts per 10^8 nucleotides).[19] Two additional studies failed to detect BPDE-*N*^{2}-dG in salivary or oral cell DNA.[20,21] Finally, a recent study failed to detect the adduct in human prostate tissue.[22]

Thus, while the diol epoxide pathway of BaP metabolic activation is widely accepted, detection of the incriminating DNA adduct in human tissues has been lacking. We addressed this problem in the study reported here by analyzing normal lung tissue obtained during lung cancer surgery. Urine samples were obtained at the same time to confirm status as cigarette smokers.

The levels of BPDE-*N*^{2}-dG adducts in human samples, when analyzed using conventional mass spectrometry,[18–22] have been typically below the limits of detection. We developed an ultra-sensitive high resolution/accurate mass (HRAM) parallel reaction monitoring (PRM) method optimizing parameters of the mass spectrometric detection, liquid chromatography, and sample preparation which when combined allowed for a dramatic improvement in detection limits of our previous HRAM MS^2 quantitation methodologies.[23–26] The focus of this effort was optimizing the sensitivity of this assay, not full method validation, using features and strategies which can potentially be applied for quantitation of other analytes present at extremely low levels. The chemical structures of the analyte and internal standards as well as the fragmentation ions used for quantitation are shown in Scheme S1.

Experimental Section

Chemicals and Supplies

$MgCl_2$ hexahydrate (BioXtra), $ZnCl_2$ (anhydrous), Trizma hydrochloride (pH 8.0, 1 M), 1-butanol (Chromasolv Plus), H_2O and CH_3CN (LC-MS Chromasolv), 2′-deoxyguanosine hydrate (dG), Amicon Ultra-0.5 (10 kDa) Centrifugal Filter Units, Benzonase DNase (endonuclease from *Serratia marcescens,* recombinant, expressed in *E. coli*), phosphodiesterase I (5′-exonuclease from *Crotalus adamanteus* venom), and alkaline phosphatase (from *Pichia Pastoris*, recombinant) were purchased from Sigma Aldrich (St. Louis, MO). DNA from calf thymus (ct-DNA) was obtained from Worthington Biochemical Corporation (Lakewood, NJ). 76-Series glass insert vials were purchased from MicroLiter (Millville, NJ). BPDE-*N*^{2}-dG (1 ng/mL) and the [$^{13}C_{10}$](±)BPDE-*N*^{2}-dG (1 ng/mL) internal standard were gifts from Dr. Robert J. Turesky, Masonic Cancer Center, University of Minnesota. The BPDE-adducted ct-DNA (1.11 adducts per 10^6 nucleotides, 1 mg DNA/mL) was a gift from Frederick A. Beland, Division of Biochemical Toxicology, National Center for Toxicological Research. The concentration of BPDE-*N*^{2}-dG and [$^{13}C_{10}$](±)BPDE-*N*^{2}-dG standards were confirmed in our laboratory using HPLC-UV detection by comparing their UV absorption to known concentrations of BaP-7,8,9,10-tetraol.

Tissue samples

After informed consent, tissue samples were obtained during surgery for lung cancer. The samples were from the margins of tumors and are considered normal. Urine samples from the same individuals were obtained prior to surgery. This study was approved by the University of Minnesota Institutional Review Board.

Analysis of urine for total cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

Total cotinine (e.g. cotinine plus its glucuronide) and total NNAL were analyzed as described previously.[27,28]

DNA hydrolysis and adduct isolation

The DNA samples and the 3 DNA hydrolysis enzymes were subjected to multiple centrifugal filtration steps using an Amicon Filter Unit to remove salts, exchange buffer and wash out contaminating small molecules which can affect MS sensitivity. Each DNA sample (352 μg DNA in 200 μl H_2O, sufficient for duplicate analyses) was centrifuged 3 times in an Amicon filter, centrifuging for 20 min at 24 °C and using 400 μL volumes of digestion buffer (5 mM Tris, 10 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ at pH 8.0) for each centrifugation. After 3 centrifugations, the 30 μL of concentrated solute in the Amicon filter was recovered by inverting the filter unit into a centrifuge tube and spinning for 2 min. The 30 μL of recovered filtrate containing the DNA was transferred to insert vials with 70 μL-of digestion buffer, enough for duplicate analysis, and stored at −20 °C.

The 3 hydrolysis enzymes (1,250 U Benzonase, 0.6 U phosphodiesterase and 6.4 U alkaline phosphatase sufficient for 200 analyses) were each centrifuged 4 times in an Amicon Filter Unit, centrifuging for 20 min at 20 °C and using 400 μL volumes of digestion buffer each time. The 30 μL of recovered concentrated solute containing the Benzonase and phosphodiesterase were each transferred to 1 mL vials with 370 μL of H_2O and stored at −20 °C. The 30 μL of recovered alkaline phosphatase was dissolved in 370 μL digestion buffer and refrigerated.

The DNA samples were hydrolyzed at 37 °C for 6 days by adding Benzonase (6.25 U in 2 μL) to an insert vial containing 50 μL of Amicon filtered DNA solution (and spiked with BPDE-adducted ct-DNA if required) and incubating for 2 days, adding phosphodiesterase (0.003 U in 2 μL) and incubating for 1 day, and finally adding alkaline phosphatase (0.032 U in 2 μL) and incubating for 3 days. After each addition of enzyme, the solution was mixed by withdrawing and dispensing the incubation mixture once using a 100 μL pipette. Details of how the enzyme amounts and appropriate hydrolysis times were determined are discussed in the Supporting Information.

The internal standard (850 amol of [$^{13}C_{10}$](±)BPDE-N^2-dG in 5 μl of 50% CH_3OH) was added to the samples by syringe after the incubation was complete. This was necessary to prevent the internal standard from binding to the 100 μL pipette tips during mixing of the DNA hydrolysis solution.

The dG concentration of the DNA digestion samples was determined by removing 2 μL, dissolving in 100 μL of H_2O and injecting 1 μL onto a Dionex UltiMate 3000 RSLCnano UPLC system with UV detection at 254 nm and a Luna C18(2) (250 x 0.5 mm, 5μm, 100A) HPLC column. The HPLC column was eluted at 15 μL/min with 2% methanol for 10 min, followed by a linear gradient to 15% methanol over 30 min. The dG peak eluted at 18 min and its concentration was determined by comparing to a standard calibration curve.

BPDE-N^2-dG was extracted into butanol by adding 80 μL of H_2O-saturated butanol, vortexing for 40 s, centrifuging to separate the layers, and removing and discarding the bottom aqueous layer using a 100 μL air-tight Hamilton syringe. The butanol layer was extracted with H_2O to remove residual salts by adding 50 μL of H_2O, vortexing for 40 s, centrifuging to separate the layers, and discarding the H_2O layer as described above. The butanol layer was dried by Speed-vac evaporation and reconstituted by vortexing in 10 μL of 10% CH_3CN. The samples were then dried by Speed-vac evaporation and reconstituted by vortexing in 3 μL of 10% CH_3CN. After spinning the samples to concentrate the liquid, the 3 μL sample was injected onto LC–ESI-MS/MS for analysis.

Spiking experiments

Two spiking experiments were performed to verify the ability of the analytical method to quantify BPDE-N^2-dG adducts at trace levels. Varying amounts of BPDE-N^2-dG synthetic standard and BPDE-adducted ct-DNA were spiked into samples containing constant amounts of ct-DNA (40 μg) and [$^{13}C_{10}$]BPDE-N^2-dG internal standard. These samples were processed and the amount of BPDE-N^2-dG quantified following the sample preparation and LC-ESI-MS/MS methodology used for analysis of the lung tissue samples.

LC-ESI-MS/MS analysis for BPDE- N^2-dG

Mass Spectrometric Parameters—The BPDE- N^2-dG concentration was measured using a Thermo Scientific Fusion Orbitrap/ion trap hybrid mass spectrometer (Thermo Fisher, San Jose, CA) in the positive ion profile mode performing parallel reaction monitoring (PRM). Parent ions of *m/z* 570.2 and *m/z* 580.2 for the analyte and internal standards, respectively, were isolated with a quadrupole isolation window of *m/z* 1.5 and HCD fragmentation setting of 15% performed in the ion routing multipole. Quantitation was performed by extracting the fragment masses of *m/z* 454.1510 ± 0.0023 (5 ppm) and *m/z* 459.1678 ± 0.0023 (5 ppm) for the analyte and internal standard, respectively. The Easy-IC internal mass calibration feature was used to assure maximum mass accuracy. Two additional fragments, *m/z* 303.1016 and *m/z* 285.0910, were also monitored as confirming ions for both the analyte and internal standard (Figure S1). The maximum Orbitrap inject times of 1000 ms and 100 ms for the analyte and internal standard, respectively, were used as were AGC target and resolution settings of 2e5 and 60,000, respectively. In addition to the PRM scanning, full scan (FS) (*m/z* 100 – 2000) data was acquired, at a resolution setting of 15,000, AGC setting of 2e5, and maximum injection time of 100 ms, to assist in method development and to assess possible differences in individual samples due to variation in sample preparation or basic sample composition. Ionization was performed using a Nanoflex nanospray ion source (Thermo Scientific) with the following parameters: spray voltage of 2200V, ion transfer tube temperature of 300 °C, and RF lens settings of 80% and 60% for PRM and FS, respectively.

Liquid Chromatography—Chromatographic separation was performed using a Dionex UltiMate 3000 RSLCnano UPLC system coupled to the nanospray ion source containing a capillary column (75 μm ID, 15 cm length, 10 μm orifice) created by hand packing a commercially available fused-silica emitter (New Objective, Woburn, MA) with XBridge BEH C18 (5 μm particle size, 130 Å pore size) stationary phase (Waters, Milford, MA). The

LC solvents were: (A) 0.05% CH_3COOH in H_2O and (B) CH_3CN. Three μL injections were performed and the initial flow rate and composition of 5% B and 600nl/min was held for 10 min to push sample out of the 5 μL autosampler loop, followed by switching the injection valve from the inject to load position to take the loop out of the flow path, and a decrease of the flow rate to 300 nL/min over the course of 1 min. This was followed by a linear gradient of the B composition over 6.5 min to 61.5% B at which point the flow rate was decreased to 150 nL/min and held at 61.5% B for 2 min. The column was re-equilibrated between injections at the initial conditions for 9 min. To assure maximum capture of the 3 μL injection from the 3 μL sample volume, the sampler sample height was set to 0.0 mm in the Xcalibur method and the sample container height offset was set to 0.0 mm in the DCMSLink Chromeleon Xpress UPLC control software.

Results

Method Sensitivity Optimization

Recently, a rigorous approach was taken to optimize the sensitivity of the LC-MS analysis of BPDE-N^2-dG.[29] Using that study as a starting point, we increased the sensitivity of the analysis for BPDE- N^2-dG by 4 to 5 orders of magnitude, allowing the detection of BPDE-N^2-dG at levels as low as 1 adduct per 10^{11} nucleotides (S/N = 2) vs the previously described detection limit of 53 adducts per 10^8 nucleotides (S/N = 6).[29] Some of this improvement in sensitivity can be accounted for by the larger amount of DNA analyzed (~40 μg vs 2 μg), however the majority of the improvement is attributable to several important features which optimized our existing HRAM MS^2 nanospray quantitation methodology[23–26] as discussed below, and can potentially be used to improve the sensitivity of other quantitative assays.

Maximizing Injection Time/Sample Preparation—Trap-based instruments have finite limits on the number of ions which can be stored prior to mass analysis such that space-charging effects do not adversely affect the resolution and mass accuracy of the detected ions. For this reason, the number of ions allowed into the trap is tightly regulated through dynamic control [e.g. automated gain control (AGC) for Thermo Scientific instrumentation] of injection times to account for varying ion fluxes such that the number of ions in the trap doesn't exceed the maximum number (AGC target value) which can be reliably measured. A maximum injection time is also set such that the trapping times, and hence the analysis rate, provide sufficient data points for quantitation or allow for rapid enough screening of analytes as they elute from the liquid chromatography column. The injection time is a critical factor in determining the limits of detection and quantitation (i.e. sensitivity) as it determines the number of ions present for spectral measurement. Therefore, the method presented here was developed to allow for the longest possible injection times (maximum injection time of 1000 ms with an AGC target value of 2e5) while still maintaining a sufficient number of chromatographic data points for quantitation. There is also a relationship between injection times and resolution settings, in terms of optimal instrument scanning efficiency, which is discussed briefly in the Supporting Information.

The first step in the acquisition of each MS^2 fragmentation spectrum for PRM detection of BPDE-N^2-dG is the isolation of the 570.2 Da parent ion by the quadrupole mass filter. The efficiency of ion transmission through the quadrupole increases with isolation width (Figure S2). Co-eluting ions within the isolation width of the quadrupole can reduce fill times below the maximum set value, especially when large maximum injection times are used. To balance the possible decrease in ion injection times with the increase in ion transmission efficiency as isolation width is increased, an isolation width value of m/z 1.5 (ions within masses within 569.45 – 570.95 Da) was used as minimal transmission loss (15%) at this value is observed. The origin of the background ions which can reduce the ion injection times is difficult to know, and therefore it is prudent to minimize the complexity of the matrix. It has been observed in a previous study[30,31] that major contributors to the ion complexity of DNA hydrolysate samples are impurities present in the hydrolysis enzymes and plasticizers found in plastic devices used in sample preparation such as solid-phase extraction cartridges and molecular weight filters. Based upon these observations, major modifications were made to the sample preparation methodology of the preceding studies.[19,29] These include purification of DNA and the hydrolysis enzymes, use of glass and stainless steel syringes, and use of 100 times less Benzonase, 17 times less phosphodiesterase and 10 times less alkaline phosphatase per μg of DNA. The dramatic reduction in enzyme levels from those typically used necessitated significantly longer hydrolysis times. Monitoring of injection times for optimization of the methodology and sample injections were done using Thermo Scientific FreeStyle software, which was preferable to Xcalibur Qualbrowser as it allows for plotting of injecting times for the entire chromatographic run. The steps taken in sample preparation allowed for injection times for detection of BPDE-N^2-dG at or close to the 1000 ms maximum value setting.

Complete Sample Injection—Typical LC-MS analyses involve injection of a fraction of the sample on-column. In our laboratory, common injection volumes are 8 μL out of a total volume of 20 μL; this permits a second injection, if necessary, with a residual of 4 μL and can be performed using default autosampler settings. While this strategy is straightforward, only 40% of the sample is actually measured which clearly limits the ultimate sensitivity of the analysis. In the analysis reported here, the DNA hydrolysate was reconstituted in 3 μL and a 3 μL injection volume was set in the method. The autosampler settings were adjusted to allow for this nearly complete volume injection and examination of the vials after injection indicated very little residual volume. Certainly a finite amount of volume remained in the vial, however we estimate it at ≤ 0.5 μL based on visual examination of the vial. In addition, inspection of the column pressure profiles occasionally revealed an initial low pressure period (typically ~1 min) indicating the injection of a small amount of air similar in value to the amount of residual liquid in the vial.

Flowrate Minimization—A typical flow rate of 300 nL/min is optimal for the chromatographic separation with the 75 μm ID column used in this study. While this type of nanospray ionization flow rate provides for efficient ionization and sampling of ions, additional sensitivity could be attained by reducing the flow rate. For this reason, we maintained a 300 nL/min flow rate for the bulk of the chromatographic separation but decreased the flow rate to 150 nL/min just prior to elution of the analyte. This resulted in a

doubling of the total ion signal of the analyte (data not shown). While the maximum signal was similar when comparing standards run at 300 nL/min throughout vs reduction to 150 nL/min prior to analyte elution, the chromatographic peak width increased such that sufficient data points could be obtained to quantify the analytes at 1000 ms injection times.

DNA hydrolysis—Complete enzymatic hydrolysis of DNA resulting in liberation of the modified nucleosides for detection is necessary if the absolute quantitation of DNA adducts is to be done. To assure this is the case, samples containing 40 μg ct-DNA were spiked with BPDE-adducted ct-DNA to assess the extent of hydrolysis of the adducted DNA bases to mononucleosides. It was found that increasing the enzymes by ten-fold resulted in negligible increase in the amounts of BPDE-N^2-dG measured. This result indicates that the hydrolysis conditions used for analysis of the lung tissue were sufficient for complete hydrolysis.

Performance of the Optimized Method—The focus of this work was to optimize detection and quantitation of BPDE- N^2-dG at very low levels. For this reason, we carefully assessed the response of the instrumentation and performance of the sample preparation methodology under these conditions. The relationship between instrument response and standards showed good linearity over the range observed in the lung samples (0 to 12 amol on-column) with an R^2 of 0.997 (Figure 1A). To measure the impact of the matrix on detection, we assessed the response of the instrument over the same range with BPDE-N^2-dG spiked into ct-DNA samples and processed following the method used to measure levels in the lung DNA samples. The measurement over the range of 0 to 12 amol (Figure 1B) gave good linearity with an R^2 of 0.964, but with significant variability as shown by the error bars (± 1 standard deviation). The offset observed in Figure 1B is due to low levels of BPDE-N^2-dG adducts present in ct-DNA. The overall analyte recovery was estimated to be 80 – 90% as determined by comparison of the internal standard signal intensities for the samples to those for the synthetic standard injections. As a more realistic simulation to the measurement in lung samples, varying amounts of BPDE-adducted ct-DNA (1.1 adducts per 10^6 nucleotides) were spiked into samples containing unmodified ct-DNA at levels of BPDE-N^2-dG covering a large range (up to 375 amol) (Figure S3) and including the lower levels of those measured in the lung DNA (Insert). Reasonably linear relationships were observed over the entire range and the lower range covering the sample levels with R^2s of 0.995 and 0.961, respectively. The discrepancy between the spiked and measured amounts of BPDE-N^2-dG is discussed in the DNA hydrolysis discussion later in the Results section. Our method attained an LOD of 1 amol of BPDE-N^2dG on-column, corresponding to 1 BPDE-N^2-dG adduct/10^{11} nucleotides (1 adduct/10 human lung cells) using an average of 43 μg of human lung DNA as measured by dG quantitation after DNA hydrolysis, in spite of ion suppression of approximately 80% determined by comparison of internal standard peak area with and without matrix.

Measured adduct levels

A unique feature of this study was collection of urine samples from the subjects to determine recent smoking status. A diagnosis of lung cancer is frequently a reason for immediate smoking cessation. Thus, many patients will quit perhaps weeks before surgery. This would have an unknown effect on DNA adduct levels since their persistence in human lung tissue is

virtually unknown. In this study, we were able to identify individuals who continued to smoke up to 1–2 days prior to surgery. As shown in Table S1, these smokers had 647–15,980 pmol/ml total cotinine and 0.84 –5.80 pmol/ml total NNAL, confirming that they smoked cigarettes virtually until their day of surgery, whereas total cotinine and total NNAL were very low or non-detectable in the non-smokers. Among the samples in which BPDE-N^2-dG was detected, there were 8 confirmed smokers, 2 of whom were male. There were 12 confirmed non-smokers, 5 of whom were male. All subjects were white.

A trace from a smoker is illustrated in Figure 2A. The retention time of the signal for *m/z* 570.2 → *m/z* 454.1487 – 454.1533 (19.12 min), corresponding to loss of deoxyribose (with transfer of H to dG-containing fragment ion) from the protonated molecular ion matches that of the internal standard, *m/z* 580.2 → *m/z* 459.1655 – 459.1701 (19.12 min) and the peaks have similar shapes although there appears to be additional signal in the analyte channel, possibly due to other PAH adducts such as those derived from benzofluoranthenes. The analyte also displays peaks at *m/z* 570.2 → *m/z* 303.1001–303.1031 corresponding to loss of dG and at *m/z* 570.2 → *m/z* 285.0896–285.0924 corresponding to loss of dG + H_2O. A trace from a non-smoker is shown in Figure 2B. Signal corresponding to the analyte is seen in the *m/z* 570.2 → *m/z* 454.1487 – 454.1533 channel and in the *m/z* 570.2 → *m/z* 303.1001–303.1031 channel, but with weaker intensity than in that of the smoker. No signal was detected in the 570.2 → *m/z* 285.0896–285.0924 channel. The non-smokers mostly had detectable signal only in the *m/z* 570.2 → *m/z* 454.1487 – 454.1533 channel. Weak signal in this channel was also detected in calf thymus DNA blanks. Typically with PRM analyses, all the abundant ions, in addition to being used for analyte identity confirmation, can be summed to provide superior limits of quantitation. For our samples, often only the most abundant ion was above the limit of detection and because of this it was decided that the quantitation would be done using only it.

The analysis of 29 human lung DNA samples resulted in 20 positive measurements with values above the LOD but below the LOQ (Table S1). Estimated average amounts of BPDE-N^2-dG in smoker and non-smoker samples were 3.1 ± 2.2 and 1.3 ± 0.8 adducts/10^{11} nucleotides, respectively. For purposes of calculating average values, each ND sample was assigned a value of 0.45 adducts per 10^{11} nt (50% of the LOD). The LOD was determined to be the lowest level measured for a sample (0.9 adducts per 10^{11} nt). Excluding ND samples, estimated average amounts of BPDE-N^2-dG in smoker and non-smoker samples were 4.0 and 1.7 adducts/10^{11} nucleotides, respectively. The calculation of Y adducts per X unmodified nucleotides is performed using the following equation:

$$Y = (A * X)/(dG * NTr)$$

where A is the number of moles of BPDE-N^2-dG adducts in the sample measured by LC-MS^2, dG is the number of moles of dG in the same sample as determined by HPLC-UV, and NTr is the ratio of total nucleotides divided by dG nucleotides (e.g. 4.8 in human DNA).[32]

The Orbitrap injection times of the analyte transition were examined at the retention times of the analyte elution, as the number of analyte ions that can be accumulated for Orbitrap detection is directly proportional to the injection time and helps define the LOD and LOQ

for a given sample. The injection times typically reached the maximum injection time of 1000 ms which was set in the instrument method.

Discussion

We report the first quantitation of BPDE-N^2-dG – the major characterized DNA adduct of the carcinogen BaP - in normal lung tissue from current smokers. The levels of BPDE-N^2-dG detected in this study, ranging from about 0.8–6.5 adducts per 10^{11} nucleotides, or about 1 – 8 amol per sample, are apparently the lowest amounts of an intact carcinogen-DNA adduct detected by mass spectrometry in free living humans, not intentionally dosed with ^{14}C-labelled compounds (e.g. for detection by accelerator mass spectrometry), that have been reported to date.[33–39] We did not perform a statistical analysis of the semi-quantitative measured values since this could imply greater accuracy of measurement than is the case, and lead to over-interpretation of the results and conclusions being drawn which may not be valid. The intent of this publication was primarily to illustrate the steps which can be taken to maximize the sensitivity for LC-MS quantitative assays, and illustrated though the detection of the ultra-trace level BPDE-N^2-dG analyte.

While the number of samples analyzed was limited and the quantitation of these extremely low adduct levels is challenging, our results nevertheless support the hypothesis that BPDE-N^2-dG is present in the lungs of smokers and at levels higher than in non-smokers. Remarkably, the estimated relative amounts in smokers and non-smokers (2.4 times higher in smokers) are consistent with the results of the NHANES study of 1-hydroxypyrene and other PAH metabolites in urine (about 2 – 3 times higher in smokers).[2,4]

The basic methodology used here is very sensitive[23–26] due to its use of HRAM MS^2 detection allowing for removal of chemical noise and efficient ionization/ion sampling through the use of nanoflow ionization. However, due to the extremely low levels of the DNA adducts, additional improvements/optimizations were performed. The first of these was maximizing the Orbitrap injection time through careful optimization of the sample preparation by purification of the DNA and hydrolysis enzymes, use of glass and stainless steel syringes, and minimizing enzyme amounts while assuring sufficient amounts were used to provide complete hydrolysis. To attain the long injection times (1000 ms) sought here to maximize sensitivity, background ions with masses within the *m/z* 1.5 mass isolation window for MS^2 fragmentation needed to be greatly reduced to avoid filling the Orbitrap before the maximum injection time is attained. Injection times were monitored using Thermo Scientific FreeStyle software which assisted in their optimization. The second step taken was the injection of as much of the sample as possible. While this may seem trivial, we are not aware of an instance in the literature where it has been done. It helps to highlight the capabilities of modern autosamplers and the sensitivity advantage of injecting as much of the sample on-column as possible. The third modification to the standard approach taken was to decrease the flow rate from 300 nL/min to 150 nL/min just prior to elution of the analyte, which provided for a doubling of the total BPDE-N^2-dG ion signal.

The developed method attained an LOD of 1 amol of BPDE-N^2-dG on-column, corresponding to 1 BPDE-N^2-dG adduct/10^{11} nucleotides (1 adduct/10 human lung cells)

using ~40 μg of human lung DNA. To our knowledge, this is the most sensitive DNA adduct quantitation method reported, exceeding the sensitivity of the ^{32}P-postlabeling assay (~1 adduct/10^{10} nucleotides). The analysis of 29 human lung DNA samples resulted in 20 positive measurements with values above the LOD but below the LOQ. Semi-quantitation of the samples was possible with average levels in smoker and non-smoker samples of 3.1 and 1.3 adducts/10^{11} nucleotides, respectively. Injection times of the analyte transition were examined at the retention times to assess the sensitivity of each individual analysis.

In our review published in 2003,[40] we stated that "conclusive evidence for the presence in humans of BPDE-N^2-dG, as the intact DNA adduct, has yet to be presented." All data summarized in that comprehensive review were based on analysis of BaP-tetraols released from DNA upon acid hydrolysis and identified by HPLC-fluorescence and GC-MS techniques, and all data from lung tissue DNA were obtained by HPLC-fluorescence techniques. Studies published since then have not significantly altered this conclusion. Beland et al used a validated LC-MS/MS method to analyze BPDE-N^2-dG in 10 normal lung tissue samples collected at autopsy and nine peripheral lung tissue samples from lung cancer patients; for seven of the lung cancer patients, DNA was also obtained from tumor tissue. Analysis of these 26 samples (48–126 μg DNA except for 2 samples with less) by LC-MS/MS, with an LOD of 0.3 BPDE-N^2-dG adducts/10^8 nucleotides when analyzing 100 μg of DNA, demonstrated that all except one were negative (<LOD), including 5 that had previously been reported to be positive by immuno-affinity chromatography-synchronous fluorescence spectroscopy, with levels ranging from 0.7–12.5 adducts per 10^8 nucleotides. Monien et al used an optimized extraction and enrichment technique coupled with a UPLC-MS/MS method to analyze 16 DNA adducts from various sources in 10 non-tumor human lung tissue samples from cancer patients.[18] Several adducts were detected and quantified, but not BPDE-N^2-dG, which was negative in all samples with detection limits of 1.0–3.0 adducts per 10^8 nucleotides. As mentioned earlier, 3 additional studies failed to detect BPDE-N^2-dG in salivary, oral cell, or prostate DNA.[20–22]

Grova et al used GC-MS/MS of released BaP-tetraols to analyze BPDE-DNA damage in white blood cell DNA from 8 humans, 3 of whom were smokers. They found a BaP-tetraol isomer above the LOQ with a mean concentration of 1.33 ± 0.52 adducts/10^8 nucleotides.[41] Rojas et al used HPLC-fluorescence to analyze released tetraols in bronchial epithelial cells from non-cancerous bronchus of 22 lung cancer subjects, and reported adduct levels in the range of 36.5–175.4 BPDE-N^2-dG adducts/10^8 nucleotides in smokers, 3 fold higher than in non-smokers.[42] Pavanello et al used HPLC-fluorescence to analyze BaP-tetraol released from DNA of lymphomonocytes and reported their presence in 248 out of 585 subjects at a mean level of 1.28 ± 2.80 adducts/10^8 nucleotides.[43]. Mensing et al also reported BaP-tetraol released from white blood cells as determined by HPLC-fluorescence with levels in the range of 1–2 adducts/10^8 nucleotides.[44]

These contrasting results between studies using mass spectrometric techniques versus those using HPLC-fluorescence to analyze BaP-tetraols released from DNA upon acid hydrolysis are difficult to reconcile. The levels of BaP-tetraols released from DNA and found by HPLC-fluorescence are consistently about 1000 times higher than we have observed here for BPDE-N^2-dG, the presumed source of the released BaP-tetraols. Similarly, the other studies

using MS approaches did not detect BPDE-N^2-dG even though their sensitivity would have been great enough to detect the adduct at levels indicated by the HPLC-fluorescence studies. Since multiple laboratories found similar results using HPLC-fluorescence, and two studies also observed released BaP-tetraol by GC-MS of their silylated derivatives,[41,45] it seems likely that the BaP-tetraol results are correct. Therefore, we speculate that there are adducts other than BPDE-N^2-dG formed by reaction of BPDE with DNA, and it is these adducts that produce the relatively high levels of BaP-tetraols upon acid hydrolysis, or that the isolated DNA used in these studies contained trace amounts of protein with high levels of bound BPDE, which was then released as tetraol by hydrolysis. Another possibility is that BaP-tetraols were intercalated into the isolated DNA and released upon hydrolysis. Further studies are necessary to resolve these issues.

We note that the estimated levels of BPDE-N^2-dG were only 2–3 times higher in smokers than in non-smokers. While this is consistent with studies of urinary metabolites of PAH such as 1-hydroxypyrene, the difference between smokers and non-smokers is far less than the difference in lung cancer risk (up to 30 times greater in smokers). There are multiple other carcinogens, co-carcinogens, and inflammatory agents in cigarette smoke that contribute to the overall effect.[33]

In summary, we describe procedural modifications and improvements that greatly improve the detectability of low levels of DNA adducts in human tissues. Applying this method to lung tissue from smokers and non-smokers, we report the first identification by mass spectrometry of BPDE-N^2-dG in human lung as the intact adduct in multiple samples, at levels ranging from 0.8 – 6.5 BPDE-N^2-dG adducts per 10^{11} nucleotides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Phillips DH. Nature. 1983; 303:468–472. [PubMed: 6304528]
2. Grainger J, Huang W, Patterson DG Jr, Turner WE, Pirkle J, Caudill SP, Wang RY, Needham LL, Sampson EJ. Environ Res. 2006; 100:394–423. [PubMed: 16225859]
3. Huang W, Grainger J, Patterson DG Jr, Turner WE, Caudill SP, Needham LL, Pirkle JL, Sampson EJ. Int Arch Occup Environ Health. 2004; 77:491–498. [PubMed: 15322857]
4. Suwan-ampai P, Navas-Acien A, Strickland PT, Agnew J. Cancer Epidemiol Biomarkers Prev. 2009; 18:884–893. [PubMed: 19258471]
5. International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 92. IARC; Lyon, FR: 2010. p. 35-818.

6. Luch, A., Baird, WM. The carcinogenic effects of polycyclic aromatic hydrocarbons. Luch, A., editor. Imperial College Press; London: 2005. p. 19-96.
7. Conney AH. Cancer Res. 1982; 42:4875–4917. [PubMed: 6814745]
8. Dipple, A., Moschel, RC., Bigger, CAH. Chemical Carcinogens, Second Edition, ACS Monograph 182. Searle, CE., editor. American Chemical Society; Washington, D.C: 1984. p. 41-163.
9. Cooper CS, Grover PL, Sims P. Prog Drug Metab. 1983; 7:295–396.
10. Xue W, Warshawsky D. Toxicol Appl Pharmacol. 2005; 206:73–93. [PubMed: 15963346]
11. Hecht SS, Hochalter JB. Mutagenesis. 2014; 29:351–356. [PubMed: 25053834]
12. Thakker, DR., Yagi, H., Levin, W., Wood, AW., Conney, AH., Jerina, DM. Bioactivation of Foreign Compounds. Anders, MW., editor. Academic Press, Inc; New York: 1985. p. 177-242.
13. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Oncogene. 2002; 21:7435–7451. [PubMed: 12379884]
14. Alexandrov LB, Ju YS, Haase K, Van Loo P, Martincorena I, Nik-Zainal S, Totoki Y, Fujimoto A, Nakagawa H, Shibata T, Campbell PJ, Vineis P, Phillips DH, Stratton MR. Science. 2016; 354:618–622. [PubMed: 27811275]
15. Lu D, Harvey RG, Blair IA, Penning TM. Chem Res Toxicol. 2011; 24:1905–1914. [PubMed: 21962213]
16. Penning TM, Burczynski ME, Hung CF, McCoull KD, Palackal NT, Tsuruda LS. Chem Res Toxicol. 1999; 12:1–18. [PubMed: 9894013]
17. Casale GP, Singhal M, Bhattacharya S, Ramanathan R, Roberts KP, Barbacci DC, Zhao J, Jankowiak R, Gross ML, Cavalieri EL, Small GJ, Rennard SI, Mumford JL, Shen M. Chem Res Toxicol. 2001; 14:192–201. [PubMed: 11258968]
18. Monien BH, Schumacher F, Herrmann K, Glatt H, Turesky RJ, Chesne C. Anal Chem. 2015; 87:641–648. [PubMed: 25423194]
19. Beland FA, Churchwell MI, Von Tungeln LS, Chen S, Fu PP, Culp SJ, Schoket B, Gyorffy E, Minarovits J, Poirier MC, Bowman ED, Weston A, Doerge DR. Chem Res Toxicol. 2005; 18:1306–1315. [PubMed: 16097804]
20. Bessette EE, Spivack SD, Goodenough AK, Wang T, Pinto S, Kadlubar FF, Turesky RJ. Chem Res Toxicol. 2010; 23:1234–1244. [PubMed: 20443584]
21. Bessette EE, Goodenough AK, Langouet S, Yasa I, Kozekov ID, Spivack SD, Turesky RJ. Anal Chem. 2009; 81:809–819. [PubMed: 19086795]
22. Xiao S, Guo J, Yun BH, Villalta PW, Krishna S, Tejpaul R, Murugan P, Weight CJ, Turesky RJ. Anal Chem. 2016; 88:12508–12515. [PubMed: 28139123]
23. Yang J, Villalta PW, Upadhyaya P, Hecht SS. Chem Res Toxicol. 2015; 29:87–95. [PubMed: 26633576]
24. Ma B, Villalta PW, Zarth A, Kotandeniya D, Upadhyaya P, Stepanov IS, Hecht SS. Chem Res Toxicol. 2015; 28:2151–2159. [PubMed: 26398225]
25. Zarth AT, Cheng G, Zhang Z, Wang M, Villalta PW, Balbo S, Hecht SS. Chem Biol Interact. 2014; 215:40–45. [PubMed: 24632417]
26. Balbo S, Villalta PW, Hecht SS. Chem Res Toxicol. 2011; 24:1729–1734. [PubMed: 21859140]
27. Carmella SG, Ming X, Olvera N, Brookmeyer C, Yoder A, Hecht SS. Chem Res Toxicol. 2013; 26:1209–1217. [PubMed: 23837805]
28. Murphy SE, Park SSL, Thompson EF, Wilkens LR, Patel Y, Stram DO, Le Marchand L. Carcinogenesis. 2014; 35:2526–2533. [PubMed: 25233931]
29. Klaene JJ, Flarakos C, Glick J, Barret JT, Zarbl H, Vouros P. J Chromatogr A. 2016; 1439:112–123. [PubMed: 26607319]
30. Balbo S, Hecht SS, Upadhyaya P, Villalta PW. Anal Chem. 2014; 86:1744–1752. [PubMed: 24410521]
31. Balbo S, Turesky RJ, Villalta PW. Chem Res Toxicol. 2014; 27:356–366. [PubMed: 24437709]
32. Bansal M. Curr Sci India. 2003; 85:1556–1563.
33. Hecht SS. Int J Cancer. 2012; 131:2724–2732. [PubMed: 22945513]
34. Singh R, Farmer PB. Carcinogenesis. 2006; 27:178–196. [PubMed: 16272169]

35. Phillips DH, Venitt S. Int J Cancer. 2012; 131:2733–2753. [PubMed: 22961407]
36. Tretyakova N, Goggin M, Sangaraju D, Janis G. Chem Res Toxicol. 2012; 25:2007–2035. [PubMed: 22827593]
37. Gavina JM, Yao C, Feng YL. Talanta. 2014; 130:475–494. [PubMed: 25159438]
38. Liu S, Wang Y. Chem Soc Rev. 2015; 44:7829–7854. [PubMed: 26204249]
39. Klaene JJ, Sharma VK, Glick J, Vouros P. Cancer Lett. 2013; 334:10–19. [PubMed: 22960573]
40. Boysen G, Hecht SS. Mutat Res. 2003; 543:17–30. [PubMed: 12510015]
41. Grova N, Salquebre G, Hardy EM, Schroeder H, Appenzeller BM. J Chromatogr A. 2014; 1364:183–191. [PubMed: 25239702]
42. Rojas M, Marie B, Vignaud JM, Martinet N, Siat J, Grosdidier G, Cascorbi I, Alexandrov K. Cancer Lett. 2004; 207:157–163. [PubMed: 15072824]
43. Pavanello S, Pulliero A, Saia BO, Clonfero E. Mutat Res. 2006; 611:54–63. [PubMed: 16978913]
44. Mensing T, Marczynski B, Engelhardt B, Wilhelm M, Preuss R, Kappler M, Angerer J, Kafferlein HU, Scherenberg M, Seidel A, Bruning T. Int J Hyg Environ Health. 2005; 208:173–178. [PubMed: 15971856]
45. Pavanello S, Favretto D, Brugnone F, Mastrangelo G, Dal Pra G, Clonfero E. Carcinogenesis. 1999; 20:431–435. [PubMed: 10190558]

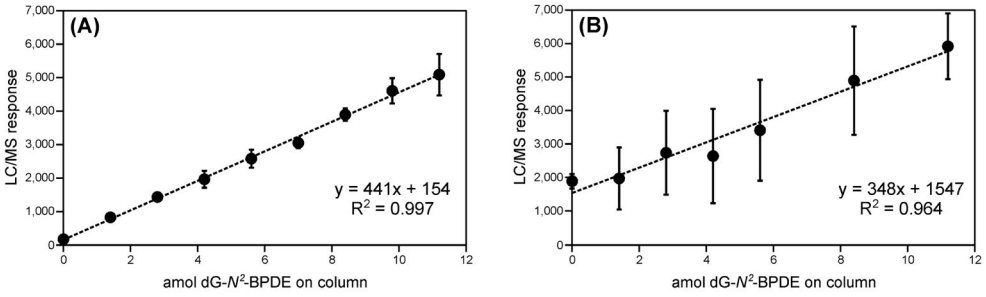


Figure 1.

Plots of the average (triplicate) ion signal response resulting from analysis of varying amounts of analyte (BPDE-N^2-dG) with constant amounts of internal standards ([$^{13}C_{10}$]BPDE-N^2-dG). Error bars represent one relative standard deviation of the triplicate measurements. Samples for (A) are neat standards in 90% H_2O, 10% ACN. Samples for (B) are from spiked ct-DNA (40 μg) processed in the same fashion as used for samples derived from lung tissue.

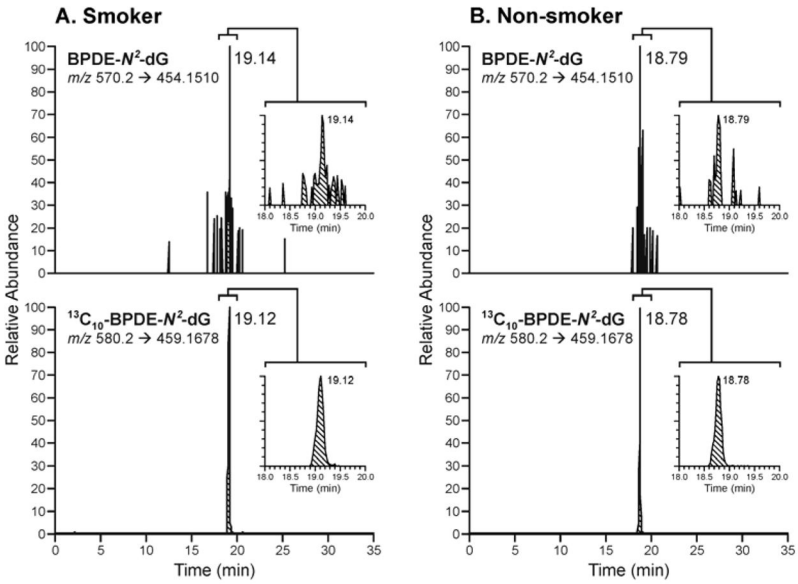
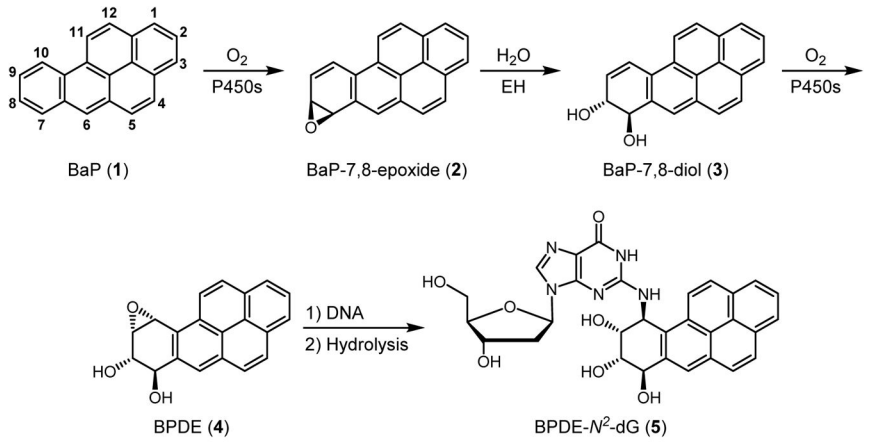


Figure 2.

Extracted ion chromatograms for the BPDE-N^2-dG analyte and [$^{13}C_{10}$]BPDE-N^2-dG internal standard ion signal with a mass tolerance of 5 ppm for a typical smoker sample (A) and no-smoker sample (B).



Scheme 1.

Metabolism of BaP to BaP-7,8-epoxide (**2**), BaP-7.8-diol (**3**), and BaP-7,8-diol-9,10-epoxide (BPDE, **4**), and reaction with DNA to give BPDE-N^2-dG (**5**).