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# NanoHPLC-nanoESI+-MS/MS quantitation of *bis*-N7-guanine DNA-DNA cross-links in tissues of B6C3F1 mice exposed to sub-ppm levels of 1,3-butadiene

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## Abstract

1,3-butadiene (BD) is an important industrial chemical and a common environmental pollutant present in urban air. BD is classified as a human carcinogen based on epidemiological evidence for an increased incidence of leukemia in workers occupationally exposed to BD and its potent carcinogenicity in laboratory mice. A diepoxide metabolite of BD, 1,2,3,4-diepoxybutane (DEB), is considered the ultimate carcinogenic species of BD due to its ability to form genotoxic DNA-DNA cross-links. We have previously employed capillary HPLC-ESI$^+$-MS/MS methods to quantify DEB-induced DNA-DNA conjugates, e.g. 1,4-*bis*-(guan-7-yl)-2,3-butanediol (*bis*-N7G-BD), 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), and *1,N*$^6$-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine (*1,N*$^6$-HMHP-dA), in tissues of laboratory mice exposed to 6.25 – 625 ppm BD (Goggin et al. *Cancer Research* 69(6), 2479–2486, 2009). However, typical BD human exposure levels are 0.01 to 3.2 ppb in urban air and 1– 2.0 ppm in an occupational setting, requiring greater detection sensitivity for these critical lesions. In the present study, a nanoHPLC-nanoESI$^+$-MS/MS method was developed for ultra-sensitive, accurate, and precise quantitation of *bis*-N7G-BD in tissues of laboratory mice treated with low ppm and sub-ppm concentrations of BD. The LOD value of the new method is 0.5 fmol/100 μg DNA, and the LOQ is 1.0 fmol/100 μg DNA, making it possible to quantify *bis*-N7G-BD adducts present at concentrations of 3 per $10^9$ nucleotides. *Bis*-N7G-BD adduct amounts in liver tissues of mice exposed to 0.5, 1.0, 1.5 ppm BD for 2 weeks were 5.7 ± 3.3, 9.2 ± 1.5, and 18.6 ± 6.9 adducts per $10^9$ nucleotides, respectively, suggesting that N7G-BD adduct formation is more efficient under low exposure conditions. To our knowledge, this is the first quantitative analysis of DEB specific DNA adducts following low ppm and sub-ppm exposure to BD.

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Supporting Information Available:
NanoHPLC-nanoESI$^+$-MS/MS standard, nanoLC-nanoESI$^+$-MS/MS traces used to determine the LOD values, and reproducibility of *bis*-N7G-BD measurements in rat liver. This material is available free of charge via the Internet at http://pubs.acs.org

## Introduction

1,3-Butadiene (BD) is an important industrial chemical commonly used in rubber and plastic manufacturing[1] and an environmental pollutant present in automobile exhaust[2] and in cigarette smoke.[3] BD is classified as human carcinogen based on epidemiological studies in occupationally exposed workers that revealed an increased incidence of leukemia and lymphohematopoietic cancers.[4–7] Furthermore, inhalation experiments with laboratory animals have shown that BD is a multi-site carcinogen, inducing lung, liver, heart stomach and other solid tumors in mice[8] and thyroid, pancreas, uterus, testes and other tissue tumors in rats.[9] BD requires metabolic activation to DNA-reactive epoxides 3,4-epoxy-1-butene (EB), 1, 2, 3, 4-diepoxybutane (DEB), and 1,2-dihydroxy-3,4-epoxybutane (EBD).[10;11] Among these, DEB is the most genotoxic, inducing base substitutions, sister chromatid exchanges, and chromosomal aberrations *in vitro* and *in vivo*.[11–13] Large interspecies differences between mutagenicity and carcinogenicity of BD in mouse and rat animal models[8;9] have been attributed to more efficient metabolic activation of BD to DEB in the mouse.[14–16] Because of its key role in carcinogenicity of BD, there is a need for sensitive biomarkers of DEB formation *in vivo.*

Georgieva et al. have developed DEB-specific biomarkers based on *N*-terminal valine hemoglobin adducts (Hb) adducts, namely N,N-[2,3-dihydroxy-1,4-butyl]valine (*pyr*-Val).[17;18] *Pyr*-Val adducts have been detected in blood of B6C3F1 mice and F344 rats exposed to BD as low as 0.5 ppm BD.[18] More recently, *Pyr*-Val adducts were observed in blood of occupationally exposed workers at concentrations of 0.08–0.86 pmol/g globin.[19] Hb-based biomarkers are valuable because protein adducts are not repaired and therefore are representative of cumulative exposure to DEB over time (the lifetime of red blood cells is ~ 120 days in humans, 45 days in mice, and 63 days in rats). However, Hb based biomarkers may not represent a biologically relevant dose of DEB since protein adducts do not lead to mutations, and DNA rather than cellular proteins is the ultimate biological target of genotoxic carcinogens such as DEB.

Our laboratory previously identified several DEB-specific DNA adducts including DNA-DNA cross-links, 1,4-*bis*-(guan-7-yl)-2,3-butanediol (*bis*-N7G-BD) and 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), and an exocyclic DEB-dA adduct, *1*,$N^6$-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine (*1*,$N^6$-HMHP-dA)(Scheme 1).[20–25] DEB-specific bifunctional DNA adducts have been quantified in tissues of laboratory rats and mice that were exposed to 6.25–625 ppm of BD by inhalation.[26] Among DEB-DNA adducts, *bis*-N7G-BD were present at the highest concentration (3.95 ± 0.89 adducts/$10^7$ nucleotides (nts) in livers of mice exposed to 625 ppm BD), followed by N7G-N1A-BD(0.27 ± 0.07 adducts/$10^7$ nts) and *1*,$N^6$-HMHP-dA (0.044 ± 0.008 adducts/$10^7$ nts).[23;26]

While our previously described capillary HPLC-$ESI^+$-MS/MS methods were adequate for detection of *bis*-N7G-BD in tissues of laboratory animals exposed to 6.25 ppm BD and higher,[26] greater sensitivity is required in order to detect DEB-DNA adducts following typical human exposures (1 ppm and lower). In the present work, we have developed and validated a nanoHPLC-nano$ESI^+$-MS/MS method for quantitative analysis of *bis*-N7G-BD *in vivo*. The sensitivity and accuracy of the new method are as follows: LOD: 0.5 fmol/100 μg DNA, LOQ: 1.0 fmol/100 μg DNA, and accuracy: 97.1 ± 6.3 % (n = 5). To our knowledge, this is the first quantitative analysis of DEB-specific DNA adducts following exposure to occupationally relevant BD concentrations (OSHA limit = 1 ppm).[27] Our results indicate that *bis*-N7G-BD formation is more efficient at low BD exposure levels (< 1.5 ppm), probably because a greater fraction of BD is metabolized to DEB. These results will be useful for human risk assessment from exposure to BD.

## Materials and Methods

### Note

DEB is a known carcinogen and must be handled with adequate safety precautions. Phenol and chloroform are toxic chemicals that should be handled in a well-ventilated fume hood with appropriate personal protective equipment.

LC-MS grade water, methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were obtained from Sigma-Aldrich (Millwaukee, WI, St. Louis, MO) with the exception of *bis*-N7G-BD and [$^{15}N_{10}$]-*bis*-N7G-BD, which were prepared in our laboratory as described elsewhere.[20;21;28]

### Animals and treatment

BD inhalation exposure experiments were performed at the Lovelace Respiratory Research Institute (Albuquerque, NM) as previously reported.[26] Briefly, B6C3F1 female mice (5 per group) were exposed to 0, 0.5, 1.0, or 1.5 ppm BD by inhalation for 2 weeks (6 h/day, 5 days/week). Animals were euthanized by cardiac puncture at the end of the exposure period. Liver tissues were collected, flash frozen, and shipped to the University of Minnesota on dry ice, where they were stored at −80 °C.

### Cell culture

V79 chinese hamster lung fibroblasts (CHL cells) were obtained from the Coriell institute (Camden, NJ, USA). Cells were grown to confluence on 15 cm dishes in Ham's F-12 modified essential Eagle's media supplemented with 9% fetal bovine serum. The cell culture was maintained in a humidified environment containing 5% carbon dioxide and 95% air at 37°C. Cells were subjected to serial passage at a dilution of 1:10 following brief exposure to porcine pancreatic trypsin and mechanical agitation. All experiments were performed on cells at a low passage number.

### DNA isolation

DNA was isolated from mouse liver tissue using NucleoBond AXG500 anion exchange cartridges (Macherey-Nagel) according to the manufacturer's instructions. DNA employed for method validation was isolated from CHL-V79 cells by standard phenol-chloroform extraction. DNA concentrations were estimated using UV spectrophotometry. DNA purity was assessed from $A_{260}/A_{280}$ absorbance ratio, which was typically between 1.7 and 1.8. DNA amounts were determined by quantitation of dG in enzymatic hydrolysates as described below.

### dG quantitation

DNA aliquots (~ 10 μg) were enzymatically digested with phosphodiesterase I (6.0 mU/μg DNA), phosphodiesterase II (8.0 mU/μg DNA), DNase (3.0 U/μg DNA), and alkaline phosphatase (6.69 U/μg DNA) in 10 mM Tris-HCl/15 mM $MgCl_2$ (pH 7.2) at 37 °C overnight. HPLC-UV analysis of dG was conducted by HPLC-UV using a Zorbax SB-C8 column (4.6 × 150 mm, 5 μm, from Agilent Technologies, Palo Alto, CA) eluted with a gradient of 150 mM ammonium acetate (A) and acetonitrile (B) as described previously.[29]

### Neutral thermal hydrolysis and isolation of *bis*-N7G-BD adducts

DNA samples (100 μg) were spiked with 50 fmol of *S,S;R,R* $^{15}N_{10}$- *bis*-N7G-BD (racemic $^{15}N_{10}$- *bis*-N7G-BD, internal standard for mass spectrometry) and incubated at 70 °C for 1 hour to release *bis*-N7G-BD from the DNA backbone as a free base conjugate. Partially depurinated DNA was removed by ultrafiltration with Nanosep 10K filters (Pall

Life Sciences, Ann Arbor, MI). The filtrates containing *bis*-N7G-BD and its internal standard were purified by offline HPLC. A Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 μm, from Agilent Technologies, Palo Alto, CA) column was eluted at 1 mL/min with a gradient of 0.4% formic acid in Milli-Q water (A) and HPLC grade acetonitrile (B). Solvent composition was maintained at 0% B for 5 min and then increased to 3% B in 10 min and further to 40% B in 5 min. The solvent composition was returned to 0% B in 5 min and maintained at 0% B for 15 min. 2′-deoxythymidine (dT) and 2′-deoxyadenosine (dA) (12.0 nmol each) were added as retention time markers, eluting at 13.7 and 18.8 min, respectively. HPLC fractions containing *bis*-N7G-BD and its internal standard (14 – 18 min, about 1.25 mL) were collected, concentrated under vacuum, and dissolved in water (25 μL) for nanoHPLC-nanoESI$^+$-MS/MS analysis.

### nanoLC-nanoESI$^+$-MS/MS analysis of *bis*-N7G-BD

A Waters nanoAcquity UPLC system (Waters Corp., Millford, MA) interfaced to a Thermo-Finnigan TSQ Quantum UltraAM mass spectrometer (Thermo Fisher Scientific Corp., Waltham, MA) was used in all analyses. HPLC solvents were 0.01% acetic acid in LC-MS grade water (A) and LC-MS grade methanol: acetonitrile (1:1) (B). Samples (8 μL) were loaded on a trapping column (Symmetry C18 nanoAcquity, 0.18 × 20 mm, Waters Corp., Millford, MA) for 1 min at 0% B at a flow rate of 10 μL/min. Chromatographic separation was achieved with a nano-HPLC column (75μm × 200 mm) manually packed with Zorbax SB-C18, 5 μm chromatographic packing (Agilent Tech. Santa Clara, CA). The column was eluted at a flow rate of 0.4 μL/min. Solvent composition was maintained at 0% B for 1 min, then linearly increased to 10% B in 2 min, further to 50% B in 8 min, and finally to 80% B in 5 min. Solvent composition returned to 0 %B for a 14 min equilibration at a flow rate of 0.4 μL/min. Under these conditions, *bis*-N7G-BD eluted as a sharp peak at ~15 min.

The triple quadrupole mass spectrometer was fitted with a nanospray source from Thermo Fisher Scientific Corp. (Waltham, MA). The instrument was operated in the positive ion ESI mode with Ar as a collision gas (1.4 mTorr). The mass spectrometer was tuned upon infusion of authentic *bis*-N7G-BD solution to achieve maximum sensitivity. Quantitative analyses were conducted in the selected reaction monitoring (SRM) mode by following the MS/MS transitions corresponding to the neutral loss of guanine from protonated molecules of the analyte (*m/z* 389.1 $[M + H]^+ \rightarrow$ *m/z* 238.1 $[M + H - Gua]^+$), and the formation of protonated guanine (*m/z* 389.1 $[M + H]^+ \rightarrow$ *m/z* 152.1 $[Gua + H]^+$). Typical instrument settings included a spray voltage of 2.0 kV, capillary temperature at 300 °C, and the collision energy at 32 and 22 V for the first and the second SRM transition, respectively. The corresponding transitions for the $^{15}N_{10}$-isotopically-labeled internal standard were *m/z* 399.1 $[^{15}N_{10}\text{-}M + H]^+ \rightarrow$ *m/z* 243.1 $[M + H - [^{15}N_5]Gua]^+$ and *m/z* 157.1 $[^{15}N_5\text{-}Gua + H]^+$. The Q1 peak width was 0.4 amu, while the peak width for Q2 was 0.7 amu. The scan width was 0.4 amu, and the scan time was 0.5 s. NanoHPLC-nanoESI$^+$-MS/MS quantitation was based on the areas of the peaks in the extracted ion chromatograms corresponding to the analyte and the internal standard.

Method standard curves were constructed by analyzing solutions containing a fixed amount of $^{15}N_{10}$-*bis*-N7G-BD (50 fmol) and increasing amounts of *bis*-N7G-BD (0.5, 1.0, 5.0, 10.0, 15.0, 25.0, and 50.0 fmol) in water (in triplicate), followed by regression analysis of the actual and the observed amounts of *bis*-N7G-BD (Figure S-1 in the Supporting Information). Solvent blanks were periodically injected to monitor for potential analyte carry-over.

### Method validation

DNA isolated from CHL V-79 cells (100 μg, in triplicate) was spiked with 0, 1, 5, 10, 15, 25 or 50 fmol of *bis*-N7G-BD and 50 fmol $^{15}N_{10}$-*bis*-N7G-BD internal standard. Following

heating at 70 °C for 1 hour (neutral thermal hydrolysis), samples were filtered through Nanosep-10K ultra- centrifugation devices. *Bis*-N7G-BD and its internal standard were purified by off-line HPLC as described above and subjected to nanoHPLC-nanoESI$^+$-MS/MS analysis by usual methods. Regression analysis was conducted to compare the measured and the spiked amounts of *bis*-N7G-BD.

### Determination of LOD/LOQ, precision, and accuracy

DNA extracted from CHL V-79 cells (100 μg) was spiked with 1, 0.75, 0.5, or 0.1 fmol of *bis*-N7G-BD and 50.0 fmol of $^{15}N_{10}$-*bis*-N7G-BD. Samples were subjected to thermal hydrolysis and ultrafiltration, purified by offline HPLC, and analyzed by nanoHPLC-nanoESI$^+$-MS/MS as described above. Method limit of quantification (LOQ) was estimated as the amount of analyte which gave a signal-to-noise ratio (S/N) greater than 10 at % CV < 15%. Method limit of detection (LOD) was determined as the amount of analyte that produced a signal-to-noise ratio > 3.

To evaluate the interday and intraday accuracy and precision of the new method, *bis*-N7G-BD (2.0 fmol) and $^{15}N_{10}$-*bis*-N7G-BD (50.0 fmol) were spiked into blank DNA isolated from CHL V-79 cells (100 μg). Samples were processed as described above and analyzed three times per day on three consecutive days. Method accuracy was calculated from the equation ($A_m/A_a \times 100\%$), where $A_m$ is the measured amount of *bis*-N7G-BD and $A_a$ is the actual amount added.

**Analysis of *bis*-N7G-BD in mouse liver samples—**DNA was extracted from livers of B6C3F1 mice exposed to 0, 0.5, 1.0 or 1.5 ppm BD by inhalation (2 weeks at 6 h/day, 5 days/week) using the methodology described above. Each exposure group contained five animals. DNA aliquots (100 μg in 200 μL water) were subjected to neutral thermal hydrolysis (70 °C for 1 hr), followed by Nanosep10K ultra filtration and offline HPLC as described above. Our previous experiments have confirmed that these conditions efficiently release *bis*-N7G-BD adducts form the DNA backbone. [21;28] *Bis*-N7G-BD adducts were quantified using the newly validated nanoHPLC-nanoESI$^+$-MS/MS method. Adduct numbers were expressed as adduct numbers per $10^7$ nucleotides. DNA amounts were determined by HPLC-UV analysis of dG in DNA hydrolysates as described above.

**Method reproducibility—**DNA was extracted from six 100 mg aliquots of liver from F344 rat treated with 62.5 ppm BD by inhalation and analyzed by the standard methodology. An additional two aliquots were extracted by the Quagen DNA extraction kit and analyzed for comparison. A good agreement was observed between these separate analyses (CV 9.4 %, Supplement S-3).

## Results

### Experimental Approach

The goal of the present study was to develop ultra-sensitive methodologies to quantify *bis*-N7G-BD adducts in tissues of mice exposed to low ppm and sub- ppm levels of BD. In our approach (Scheme 2), genomic DNA is spiked with [$^{15}N_{10}$]-*bis*-N7G-BD internal standard, and *bis*-N7G-BD lesions are released from the DNA backbone by neutral thermal hydrolysis. Our previous results indicate that heating at 70 °C for 1 h results in a quantitative release of *bis*-N7G-BD cross-links.[21;26] Following the removal of partially depurinated DNA via ultrafiltration, *bis*-N7G-BD and its internal standard are isolated from the bulk of the sample matrix using off-line HPLC. HPLC fractions containing the analyte and its internal standard are then dried, reconstituted in buffer, and subjected to nanoHPLC-nanoESI$^+$-MS/MS analysis with a triple quadrupole mass spectrometer. Our new method

employs a flow rate of 0.4 μL/min and nanoESI MS/MS detection as compared to the earlier capillary HPLC-ESI-MS/MS method that uses a flow rate of 10 μL/min.[26]

**nanoHPLC-nanoESI$^+$-MS/MS method development and validation—**MS/MS fragmentation pattern of *bis*-N7G-BD in a triple quadrupole mass spectrometer is characterized by predominant product ion peaks at *m/z* 238.1 corresponding to neutral loss of guanine base from protonated molecules of the adduct $[M + H - Gua]^+$.[21] Another abundant fragment at *m/z* 152.1 corresponds to protonated guanine ions $[Gua + H]^+$ (Figure 1). The corresponding fragments for the corresponding $^{15}N_{10}$ isotopically labeled internal standard are *m/z* 243.1 $[M + H - [^{15}N_5]Gua]^+$ and *m/z* 157.1 $[^{15}N_5\text{-}Gua + H]^+$.

Our quantitative method for *bis*-N7G-BD is based on selected reaction monitoring of the MS/MS transitions *m/z* 389.1 $[M + H]^+ \rightarrow$ *m/z* 238.1 $[M + H - Gua]^+$ and *m/z* 389.1 $[M + H]^+ \rightarrow$ *m/z* 152.1 $[Gua + H]^+$. The corresponding transitions for the $^{15}N_{10}$-isotopically-labeled internal standard are *m/z* 399.1 $[^{15}N_{10}\text{-}M + H]^+ \rightarrow$ *m/z* 243.1 $[M + H - [^{15}N_5]Gua]^+$ and *m/z* 157.1 $[^{15}N_5\text{-}Gua + H]$. A sum of the two transitions is used for quantitation to improve sensitivity.

To optimize analyte separation from interfering components of the biological matrix, the new method employs an offline HPLC sample cleanup. dT and dA are spiked in each sample as retention time markers, eluting at 13.7 and 18.8 min, respectively, while the retention time of *bis*-N7G-BD and its internal standard is ~16 min. HPLC fractions containing *bis*-N7G-BD and $^{15}N_{10}$-bis-N7G-BD (14 – 18 min, about 1.25 mL) were collected and concentrated for nanoLC-nanoESI$^+$-MS/MS analysis.

We have chosen nanospray LC-MS method over conventional capillary flow LC-MS because of its greater sensitivity. When HPLC flow rate is reduced to < 1 μL/min, droplet formation in the electrospray source occurs more readily, requiring only the applied voltage (typically 2000V) and no nebulizing gas to generate a spray.[30] The efficiency of electrospray ionization is greatly improved under nanoflow conditions, because a smaller amount of mobile phase passes through the emitter, producing fine aerosol droplets. Consequently, the stability of ion spray, and therefore MS signal, is improved, thus leading to improved detection sensitivity.[31;32] As an additional benefit, nanoHPLC methods consume very small amounts of solvents as compared to conventional capillary flow LC MS methods. Several laboratories, including ours, have previously reported the use of nanospray for sensitive quantitation of DNA adducts.[33–37]

Several HPLC stationary phases were evaluated during the nanoHPLC method development, including Symmetry C18 (Waters Corp., Millford, MA), Luna C18 (Phenomenex, Torrance, CA), HILIC-$NH_2$ (Phenomenex, Torrance, CA), Synergi Polar RP and Hydro RP (Phenomenex, Torrance, CA). The best analyte retention and peak shape were obtained with Zorbax SB-C18 stationary phase (Agilent Technologies, Santa Clara, CA). We found that the best sensitivity was achieved with a gradient of 0.01% aqueous acetic acid (solvent A) and acetonitrile: methanol (1:1, solvent B). All solvents were LC-MS grade. Using a linear gradient between 10% and 100% B, *bis*-N7G-BD adducts eluted at 14.5 min and were resolved from any impurities present in DNA hydrolysates (Figure 2).

**Method Validation—**The limit of detection of the nanoHPLC-nanoESI$^+$-MS/MS method was estimated as 50 amol *bis*-N7G-BD on column, a significant increase in sensitivity as compared to our previous capillary HPLC-ESI$^+$-MS/MS method.[26] To validate the new methodology, blank DNA isolated from Chinese hamster lung fibroblasts (100 μg) was spiked with known amounts of *bis*-N7G-BD and corresponding $^{15}N_{10}$-labelled internal standard, followed by sample processing and nanoHPLC-nanoESI$^+$-MS/MS analysis as

described above. An excellent correlation was observed between the expected and observed concentrations of *bis*-N7G-BD, with an $R^2$ value of 0.999 (Figure 3). We found that the method's lower limit of quantitation (S/N ratio of 10 or better) was 1.0 fmol *bis*-N7G-BD in 0.1 mg DNA (3 *bis*-N7G-BD adducts per $10^9$ nts), and the LOD value was 0.5 fmol/0.1 mg DNA. The LOD value for pure standard of *bis*-N7G-BD was 0.1 fmol (Figure S-2). Method accuracy (n = 5) and precision (n = 3) were determined for replicates of *bis*-N7G-BD (2.0 fmol) spiked into 0.1 mg of DNA. Method accuracy was determined to be 97.1 ± 6.3 % (n = 5) and the interday and intraday precision were less than 8 % RSD (n = 3) (Table 1).

**Analysis of bis-N7G-BD in mouse liver DNA—**The newly validated quantitative nanoHPLC-nanoESI$^+$-MS/MS method was employed to analyze the formation of *bis*-N7G-BD adducts in liver DNA of B6C3F1 mice that were exposed to 0.5 – 1.5 ppm BD by inhalation for 2 weeks. Representative extracted ion chromatogram from nanoHPLC-nanoESI$^+$-MS/MS analysis of *bis*-N7G-BD in liver DNA samples from a mouse exposed to 1.0 ppm BD is shown in Figure 2. We found that DNA extracted from tissues of mice treated with 0.5 ppm BD for 2 weeks contained 5.7 ± 3.3 *bis*-N7G-BD adducts per $10^9$ normal nucleotides (N = 4), while animals exposed to 1.0 ppm BD contained 9.2 ± 1.5 *bis*-N7G-BD adducts per $10^9$ nts (N = 5) and those exposed to 1.5 ppm BD contained 18.6 ± 6.9 *bis*-N7G-BD adducts per $10^9$ normal nucleotides (N = 5). DNA of control animals (N = 5) did not contain detectable amounts of *bis*-N7G-BD (Figure 4).

## Discussion

A potent human and animal carcinogen, BD is metabolically activated to DEB, which reacts with DNA to form DNA-DNA cross links, e.g. 1,4-*bis*-(guan-7-yl)-2,3-butanediol (*bis*-N7G-BD).[20–23] If not repaired, the interstrand *bis*-N7G-BD adducts can block DNA replication and transcription and lead to genotoxicity, while the corresponding intrastrand lesions are likely to be mutagenic.[28] It is important to determine the molecular dose of DEB following BD exposure because of its central role in BD-mediated adverse health effects. In particular, DEB is 100–200-fold more mutagenic than other epoxide metabolites of BD,[11] and is thought to be responsible for most of the genotoxic effects of BD at low exposure concentrations (< 62.5 ppm BD).[12]

*bis*-N7G-BD lesions have been previously identified and quantified in laboratory animals exposed to relatively high concentrations of BD 6.25–625 ppm.[26] However, DEB-specific DNA adducts have not been previously analyzed in animals subjected to BD exposures approaching the human Occupational Safety and Health Administration (OSHA) time weighted average levels (1 ppm).[27] Ambient BD concentrations in monomer and polymer work places range from 0.3 to 3 ppm. New and improved bioanalytical methods were required in order to quantify *bis*-N7G-BD adducts in tissues following low ppm and sub-ppm exposures.

In the present study, an ultra sensitive nanoHPLC-nanoESI$^+$-MS/MS was developed for quantitation of *bis*-N7G-BD in liver DNA of mice exposed to sub-ppm levels of BD. Capillary flow HPLC-ESI-MS based methods typically employed for DNA adduct analysis are limited in their sensitivity due to the presence of relatively large amounts of solvent, which reduces the efficiency of ion formation in the ESI source. In contrast, nanospray analysis is characterized by more efficient ionization and improved sensitivity.[30;38] Therefore, we have adopted a nanospray LC-MS/MS methodology for quantitative analysis *bis*-N7G- BD *in vivo*. Our new analytical method is more sensitive than our previous capillary HPLC-ESI-MS/MS methodology (LOQ, 1.0 fmol) because of an improved sample clean up by offline HPLC and the inherent advantages of nanospray HPLC-ESI-MS/MS.

NanoHPLC-nanospray MS/MS methodology has been recently employed for the detection of 7-ethylguanine adducts in leukocyte DNA isolated from smokers and non smokers.[36] Solid phase extraction was employed for sample cleanup following DNA hydrolysis, and MS analyses were conducted with an Orbitrap LTQ Velos mass spectrometer using an accurate mass mode.[36] The LOD and LOQ values of the method were reported as 0.1 fmol on column and 8 fmol per 180 μg DNA, respectively.[36] Zarbl et al. employed nanoHPLC-nanospray MS to detect and quantitate DNA adducts in bronchial epithelial cells treated with $10^{-5}$ – $10^{-8}$ M concentrations of the N-hydroxy- 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a potent food carcinogen present in overcooked meat.[37]

In another recent study, Chen et al. employed nanoHPLC-nanospray MS/MS technique to analyze 1,$N^2$-propano-2′-deoxy-guanosine adducts derived from acrolein (AdG) and crotonaldehyde (CdG) in various human DNA samples from saliva, human placenta and blood leukocytes.[33–35] Three lipid peroxidation-related etheno adducts:1,$N^6$-etheno-2′-deoxyadenosine (εdAdo), 3,$N^4$-etheno-2′-deoxycytidine (εdCyt), and 1,$N^2$-etheno-2′-deoxyguanosine (1,N2- εdGuo) were also analyzed by nanoHPLC-nanospray MS/MS.[34] The limits of quantification per 5 μg of DNA were reported as 0.31 fmol, 1.5 fmol, 0.36 fmol, 2.0 fmol, and 1.7 fmol for AdG and CdG, εdAdo, εdCyd, and 1,$N^2$- εdGuo, respectively.[33]

Our nanoHPLC-nanospray MS/MS methodology for *bis*-N7G-BD in tissue DNA described herein is comparable to previously reported nanoHPLC-nanospray MS/MS methods in sensitivity (LOD: 0.5 fmol and LOQ: 1.0 fmol in 100μg DNA), accuracy, and precision (Table 1). With this methodology, it is possible to detect 3 *bis*-N7G-BD adducts per $10^9$ nucleotides. Since DNA-DNA cross-links are less abundant in DNA than DNA monoadducts such as 7-ethylguanine, an offline HPLC sample cleanup was required to provide a higher degree of separation than can be afforded by solid phase extraction. While such a cleanup step increases the overall analysis time, minimal operator involvement is required with the use of an automated HPLC system incorporating an HPLC autosampler and a fraction collector.

Using the validated nanoHPLC-nanospray MS/MS method, *bis*-N7G-BD adduct amounts were determined in livers of B6C3F1 mice following a 2 week exposure to 0.5, 1.0, or 1.5 ppm BD (6 h/day, 5 days/week, see Figure 4). No *bis*-N7G-BD was detected in liver DNA of control mice (N = 5) which have not been exposed to BD. A comparison of the numbers of *bis*-N7G-BD adducts/$10^7$ nucleotides/ppm of BD under low ppm exposure conditions determined in the present study to our earlier data for animals exposed to higher BD concentrations (6.25–625 ppm)[26] shows that there is higher ratio of *bis*-N7G-BD adduct numbers per ppm of BD at low levels of BD exposure as compared to BD exposures greater than 62.5 ppm (Figure 5). Table 2 provides a comparison of *bis*-N7G-BD adduct levels per unit dose of BD to *pyr*-Val adducts (picomoles per gram of globin) per ppm of BD exposure in female mice. Taken together, our results indicate that *bis*-N7G-BD adduct formation is more efficient at low concentrations of BD (≤ 6.25 ppm) as compared to higher exposure concentrations (≥ 62.5 ppm), probably a result an increased bioactivation of BD to DEB under low exposure conditions. These results are relevant because typical occupational or environmental exposure of humans to BD is 2 ppm or less.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## List of Abbreviations

| | |
|---|---|
| **BD** | 1,3-butadiene |
| ***bis*-N7G-BD** | 1,4-*bis*-(guan-7-yl)-2,3-butanediol |
| **CHL** | Chinese hamster Lung |
| **DEB** | 1,2,3,4-diepoxybutane |
| **HPLC-ESI$^+$-MS/MS** | liquid chromatography-electrospray ionization tandem mass spectrometry |
| **N7G-N1A-BD** | 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol |
| ***1,N$^6$*-γ-HMHP-dA** | *1,N$^6$*-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-dA |
| ***1,N$^6$*-α-HMHP-dA** | *1,N$^6$*-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine |
| **SRM** | selected reaction monitoring |
| ***pyr*-Val** | N,N[2,3-dihydroxy-1,4-butyl]valine |
| **THB-Val** | N-[2,3,4-trihydroxy-butyl]valine |
| **EBD** | 1,2-dihydroxy-3,4-epoxybutane |

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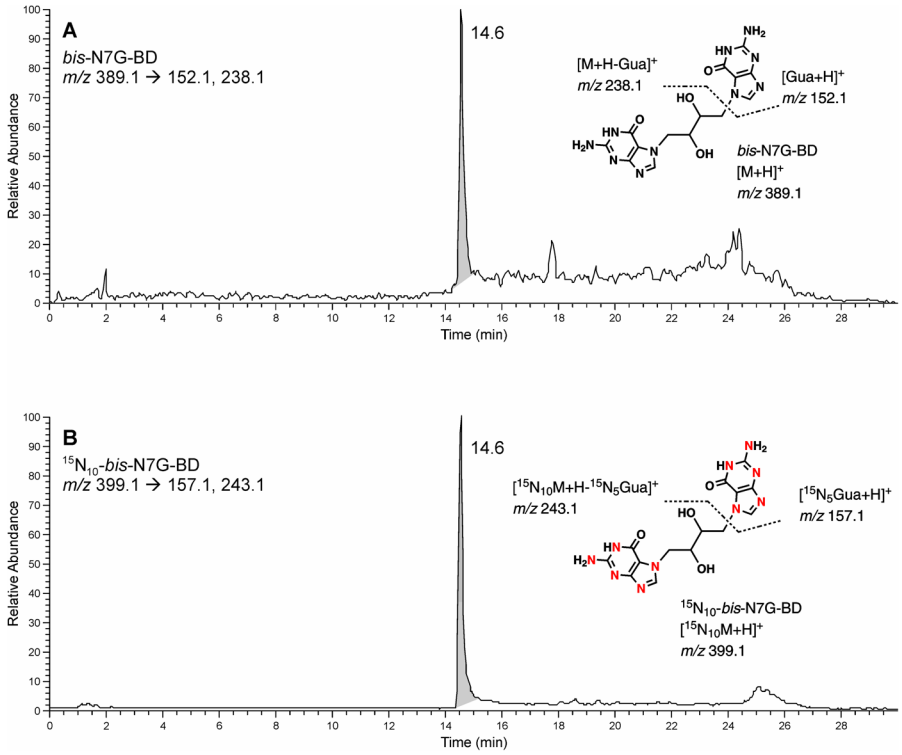



**Figure 1.**

NanoLC-nanoESI$^+$-MS/MS analysis of a mixture of pure *bis*-N7G-BD (1.0 fmol) and $^{15}N_{10}$-*bis*-N7G-BD (10 fmol).

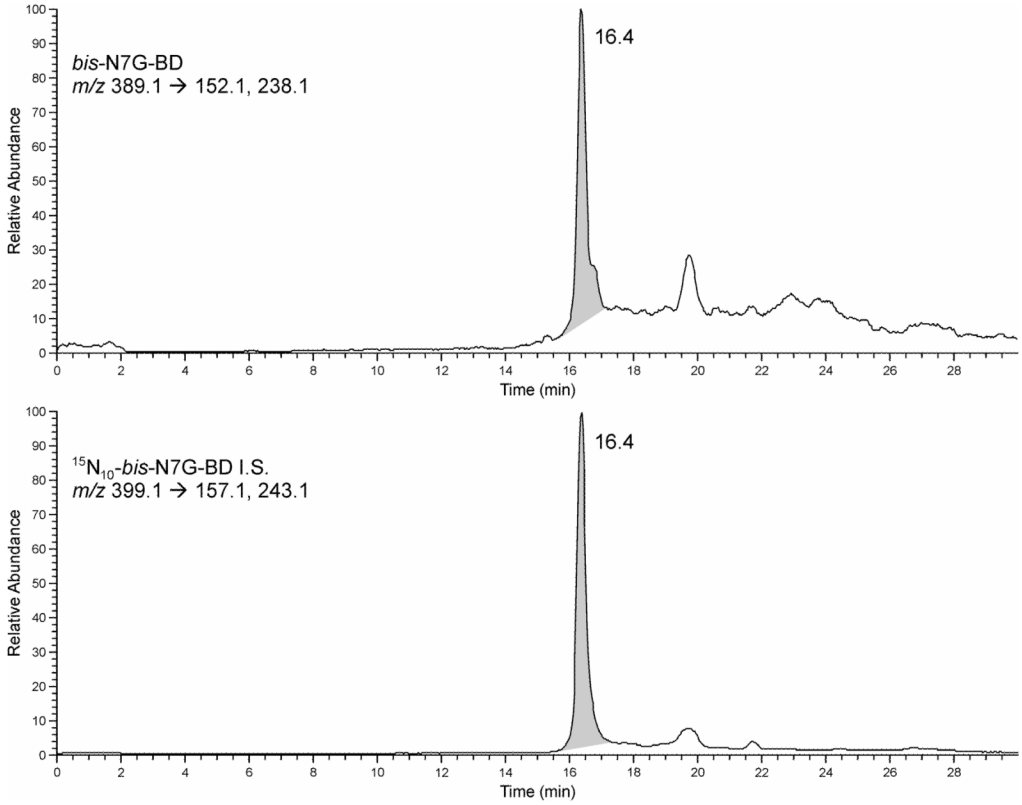



**Figure 2.**
NanoHPLC-nanoESI$^+$-MS/MS analysis of *bis*-N7G-BD in a sample of liver DNA from a mouse exposed to 1.0 ppm BD for 2 weeks.

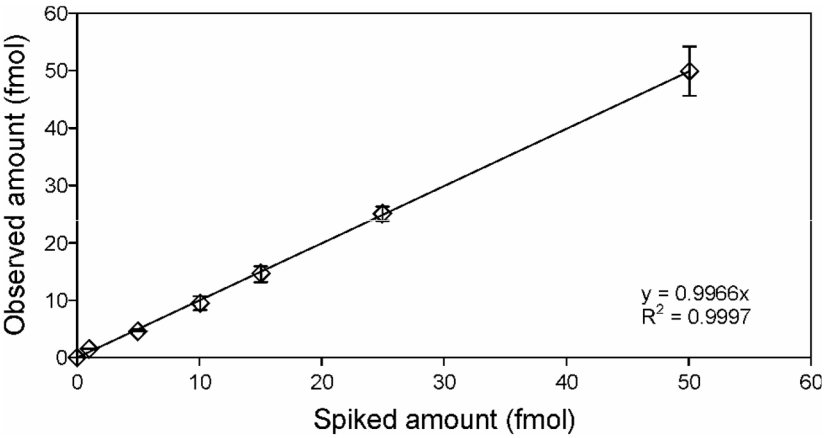



**Figure 3.**
NanoHPLC-nanoESI$^+$-MS/MS method validation curve for *bis*-N7G-BD spiked into blank DNA (0.1 mg).

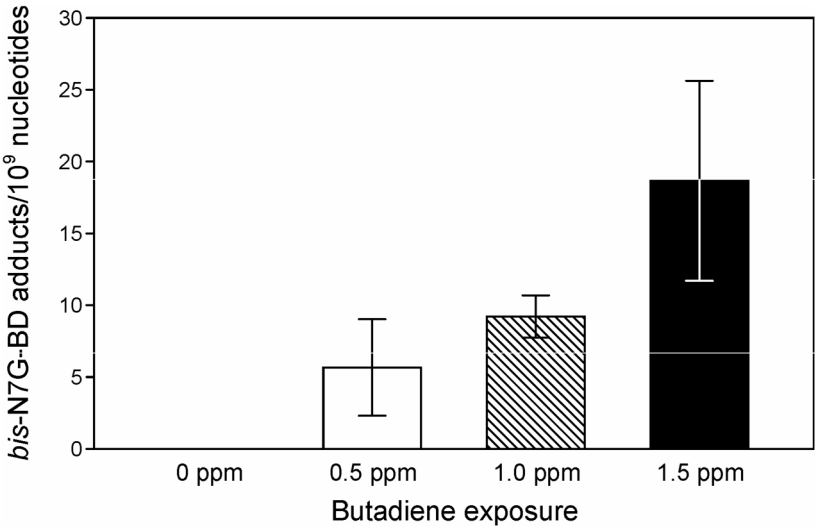



**Figure 4.**
Concentrations of *bis*-N7G-BD adducts in liver tissues of B6C3F1 female mice (4–5 per group) exposed to 0, 0.5, 1.0, or 1.5 ppm BD by inhalation for 2 weeks (6 h/day, 5 days/week).

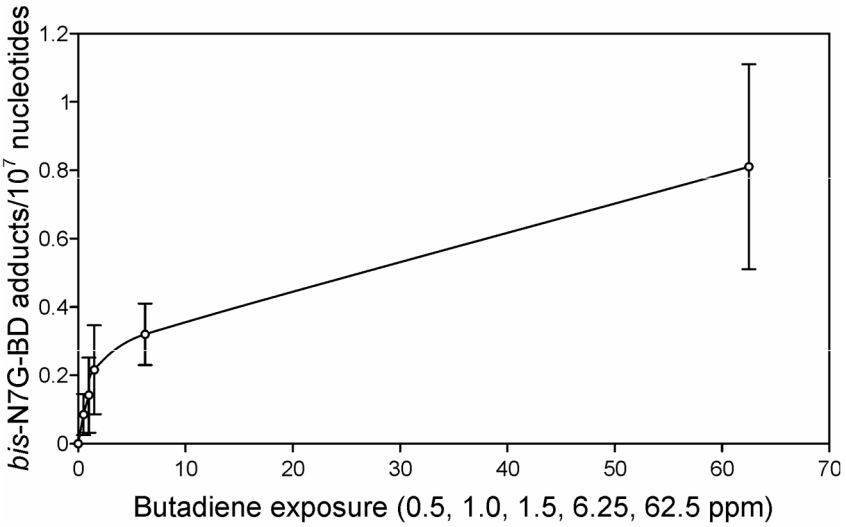
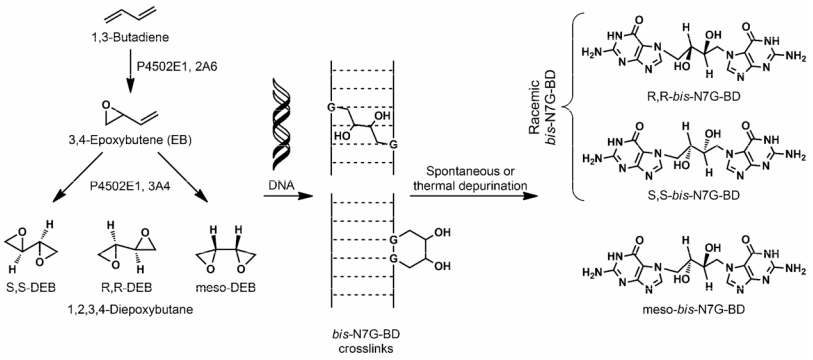
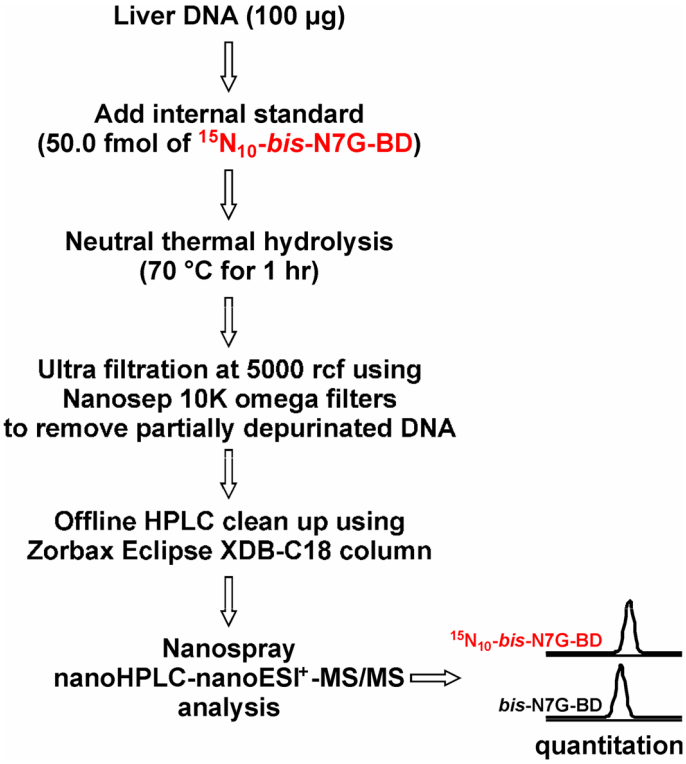



**Figure 5.**

Dose-dependent formation of *bis*-N7G-BD in liver DNA of female B6C3F1mice exposed to BD for 2 weeks by inhalation (6 h/day, 5 days/week). The 6.25 and 62.5 ppm values were taken from our earlier publication.[26]




**Scheme 1.**
Metabolism of 1,3-butadiene to DEB and its reactions with DNA to form *bis*-N7G-BD adducts.




**Scheme 2.**
*Bis*-N7G-BD analytical procedure

## Table 1

Accuracy and precision of nanoHPLC-nanoESI$^+$-MS/MS analysis for *bis*-N7G-BD (2 fmol) spiked into blank DNA (0.1 mg).

| | | |
|---|---|---|
| **Day 1** | **Mean** | 1.9 |
| | **RSD (%)** | 5.4 |
| | **Accuracy(%)** | 93.5 |
| | **N** | 3 |
| **Day 2** | **Mean** | 2.1 |
| | **RSD (%)** | 6.7 |
| | **Accuracy(%)** | 106.0 |
| | **N** | 3 |
| **Day 3** | **Mean** | 2.2 |
| | **RSD (%)** | 0.9 |
| | **Accuracy(%)** | 108.1 |
| | **N** | 3 |
| **Interday** | **Mean** | 2.1 |
| | **RSD (%)** | 7.9 |
| | **Accuracy(%)** | 102.6 |
| | **N** | 9 |

## Table 2

Efficiency of formation of *bis*-N7G-BD adducts in mouse liver DNA (DNA adducts per exposure level) and *pyr*-Val adducts (hemoglobin adducts per exposure level) in mice exposed from sub-ppm to high levels of BD for 2 weeks (6 h/day, 5 days/week).

| BD exposure (ppm) | *Bis*-N7G-BD adducts per $10^7$ nucleotides/ppm BD | *Pyr*-Val adducts, picomoles per gram of globin/ppm of BD[18] |
|---|---|---|
| 0 | ND[a] | ND |
| 0.5 | 0.170 | 21.0 |
| 1.0 | 0.142 | 20.0 |
| 1.5 | 0.144 | 26.0 |
| 6.25[b] | 0.051[b] | 12.0 |
| 62.5[b] | 0.013[b] | 6.7 |
| 200[b] | 0.009[b] | 3.7 |
| 625[b] | 0.006[b] | 2.5 |

[a] ND, not detected

[b] From our previous study.[26]