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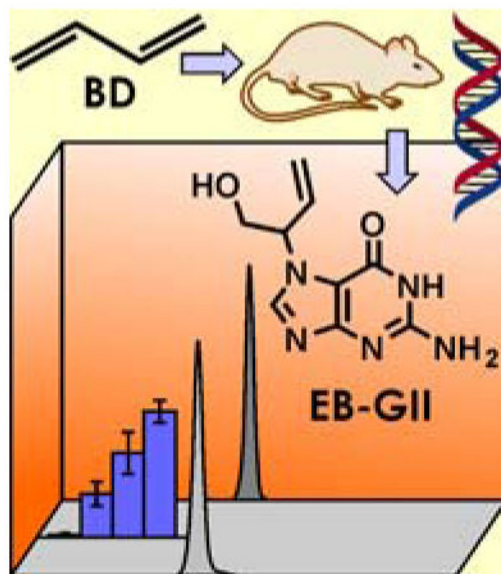
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J Am Soc Mass Spectrom. 2014 July ; 25(7): 1124–1135. doi:10.1007/s13361-014-0916-x.**NanoLC/ESI⁺ HRMS³ Quantitation of DNA Adducts Induced by 1,3-Butadiene****Dewakar Sangaraju[†], Peter W. Villalta[†], Susith Wickramaratne[†], James Swenberg[‡], and Natalia Tretyakova^{†,*}**[†]Department of Medicinal Chemistry and the Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455[‡]Department of Environmental Sciences and Engineering, The University of North Carolina, Chapel Hill, NC, USA**Abstract**

Human exposure to 1,3-butadiene (BD) present in automobile exhaust, cigarette smoke, and forest fires is of great concern because of its potent carcinogenicity. The adverse health effects of BD are mediated by its epoxide metabolites such as 3,4-epoxy-1-butene (EB), which covalently modify genomic DNA to form promutagenic nucleobase adducts. Because of their direct role in cancer, BD-DNA adducts can be used as mechanism-based biomarkers of BD exposure. In the present work, a mass spectrometry-based methodology was developed for accurate, sensitive, and precise quantification of EB-induced N-7-(1-hydroxy-3-buten-2-yl) guanine (EB-GII) DNA adducts *in vivo*. In our approach, EB-GII adducts are selectively released from DNA backbone by neutral thermal hydrolysis, followed by ultrafiltration, offline HPLC purification, and isotope dilution nanoLC/ESI⁺-HRMS³ analysis on an Orbitrap Velos mass spectrometer. Following method validation, EB-GII lesions were quantified in human fibrosarcoma (HT1080) cells treated with micromolar concentrations of EB and in liver tissues of rats exposed to sub ppm concentrations of BD (0.5–1.5 ppm). EB-GII concentrations increased linearly from 1.15±0.23 to 10.11±0.45 adducts per 10⁸ nucleotides in HT1080 cells treated with 0.5–10 μM DEB. EB-GII concentrations in DNA of laboratory rats exposed to 0.5, 1.0 and 1.5 ppm BD were 0.17±0.05, 0.33±0.08, and 0.50±0.04 adducts per 10⁸ nucleotides, respectively. We also used the new method to determine the *in vivo* half-life of EB-GII adducts in rat liver DNA (2.20±0.12 days) and to detect EB-GII in human blood DNA. To our knowledge, this is the first application of nanoLC/ESI⁺-HRMS³ Orbitrap methodology to quantitative analysis of DNA adducts *in vivo*.

Graphical Abstract

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Introduction

Humans are exposed to numerous exogenous and endogenous chemicals present in automobile exhaust, urban air, water, food, and cigarette smoke [1–3]. Among these is 1,3-butadiene (BD), a prominent industrial and environmental pollutant classified as a known carcinogen [4–7]. Human exposure to BD is due to its presence in automobile exhaust (20–60 ppb) [8], cigarette smoke (8.5–48.2 $\mu\text{g}/\text{cigarette}$) [9], wood fires (0.14–0.17 ppb) [10], and occupational sources in BD monomer and polymer industries [11–13].

BD undergoes cytochrome P450-mediated metabolic activation to three epoxide metabolites: 3,4-epoxy-1-butene (EB), 1,2,3,4-diepoxybutane (DEB), and 1,2-dihydroxy-3,4-epoxybutane (EBD) (Scheme 1) [14]. These electrophilic epoxides are held responsible for the carcinogenicity and mutagenicity of BD because of their propensity to react with DNA to form covalent nucleobase adducts [15–19], which can cause DNA polymerase errors [20–23]. Sensitive and specific quantitative methods for BD-DNA adducts *in vivo* are needed because they can be used in human cancer risk assessment [24, 25].

3,4-Epoxy-1-butene (EB) is the second most abundant epoxide formed upon metabolic activation of BD. EB reacts with the N7-position of guanine in DNA to form N-7-(2-hydroxy-3-buten-1-yl) guanine (EB-GI) and N-7-(1-hydroxy-3-buten-2-yl) guanine (EB-GII) adducts (Scheme 1) [19, 26]. EB-GI and EB-GII can be used as biomarkers of BD exposure and bioactivation, along with the corresponding hemoglobin adducts (N-(2-hydroxy-3-buten-1-yl)-valine (HB-Val)) [27] and urinary metabolites (2-(N-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(N-acetyl-L-cystein-S-yl)-2-hydroxybut-3-ene (MHBMA) [28]. While globin adducts and urinary metabolites have been extensively used as biomarkers of BD exposure and metabolic activation [27–32], one important advantage of DNA adducts is that they represent the biologically relevant dose of BD and thus are directly associated with BD-mediated mutagenesis and cancer.

In the present study, a nanoHPLC-high resolution tandem mass spectrometry (nanoLC/ESI⁺-HRMS³) methodology utilizing an Orbitrap Velos mass spectrometer was developed to allow for sensitive and specific quantitation of EB-GII adducts *in vivo*. EB-GII adducts were quantified by isotope dilution with the corresponding ¹⁵N-labeled internal standard. The new method was fully validated and applied to determine the concentrations of EB-GII in human fibrosarcoma (HT1080) cells treated with increasing concentrations of EB (50 nM to 10 μM), in liver tissues of laboratory rats exposed to BD by inhalation, and in human blood DNA.

Experimental

Note: *EB is a known carcinogen and must be handled with adequate safety precautions. Phenol and chloroform are toxic chemicals which should be used only in a well-ventilated fume hood with appropriate personal protective equipment.*

Puregene DNA purification reagents were obtained from Qiagen (Valencia, CA). LC-MS grade water, methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were obtained from Sigma-Aldrich (Millwaukee, WI, St. Louis, MO). Nonsmoker blood buffy coat fractions were purchased from BioChemed (Winchester, VA). Blood samples from known smokers and nonsmokers were acquired from the NCI CRCHD repository (PI: Dr. Peter Shields).

Synthesis of EB-guanine standards and the corresponding ¹⁵N₅ internal standards

EB-GI and EB-GII were prepared as previously reported [26, 33]. Briefly, dG (105 mg, 0.393 mmol) was reacted with 3,4-epoxy-1-butene (EB) (266 mg, 3.93 mmol) in 5 mL of glacial acetic acid at 50 °C for 5 h. The reaction mixture was precipitated with one volume of acetone and 4 volumes of ether, and the resulting white precipitate was air dried and dissolved in 1.5 mL of 1N HCl. The reaction mixtures was heated at 80 °C for 5 h to induce depurination, cooled down, and neutralized with NaOH. EB-GI and EB-GII were isolated by reverse phase HPLC on a Synergi Hydro RP 80R semipreparative column (250 mm × 10 mm, 4μ) (Phenomenex, Torrance, CA) using a gradient of water and acetonitrile at 2 mL/min. Under these conditions, EB-GI and EB-GII eluted at 22.5 and 24.1 min, respectively. Both adducts were characterized by UV spectrophotometry, ¹H NMR, and MS/MS, which were consistent with literature data [26]. ¹⁵N₅-EB-GI and ¹⁵N₅-EB-GII were prepared analogously starting with ¹⁵N₅-dG. Molar concentrations of EB-G and ¹⁵N₅-EB-G standard solutions were determined by UV spectrophotometry using the molar extinction coefficient (ε) of 8030 M⁻¹ cm⁻¹ at 284 nm at neutral pH [33]. Isotopic purity of ¹⁵N₅-EB-GI and II as determined by mass spectrometry was 99.97 and 99.93 %, respectively.

HT1080 cell culture experiments

Human fibrosarcoma cells (HT1080) were grown in Dulbecco's modified Eagle's media supplemented with 9% fetal bovine serum (Life technologies, Grand Island, NY). Cells were cultured in a humidified atmosphere of 5% carbon dioxide and 95% air, at 37 °C. HT1080 cells were plated into 15 cm dishes using Dulbecco's modified Eagle's medium containing 9% FBS and permitted to adhere overnight at 37 °C. On the following morning, cells (in

duplicate) were treated with increasing concentrations of EB (0, 500 nM, 1 μ M, 5 μ M or 10 μ M) for 3 h at 37 °C. The EC₅₀ for EB in HT1080 cells treated with 0.25 to 50 μ M for 48 h was 13.8 μ M as determined by the alamar blue assay (Life technologies, Grand Island, NY). Control and treated cells (N = 2) were harvested, washed with ice cold phosphate-buffered saline (PBS), and suspended in 5mL of PBS for DNA extraction as described below.

Animals and Treatment

In the first study, male and female F344 rats (N = 3 per group) were exposed to 0.5, 1.0, or 1.5 ppm for 2 weeks (6 h/day, 5 days/week) using whole-body exposure chambers at the Lovelace Respiratory Research Institute (LRRI, Albuquerque, NM) as reported previously [34].

In a separate study, female F344 rats (3 per group) were exposed to 1250 ppm BD by whole-body exposure chamber inhalation for 10 days (7 h/day) at LRRI. The animals were sacrificed either immediately at the end of the exposure period or 1, 3, or 6 days post exposure as reported previously [16]. Liver tissue was collected, flash frozen, and shipped to the University of MN on dry ice, where they were stored at – 80 °C until DNA extraction. All protocols are approved by the Institutional Animal Care and Use Committee at LRRI.

Human study subjects

Human smoker and nonsmoker blood buffy coat (white blood cells) samples were obtained from the National Cancer Institute. These samples were a part of the NCI 3ARM study supported by National Cancer Institute contract HHSN261200644002 (Laboratory Assessment of Tobacco Use Behavior and Exposure to Toxins Among Users of New Tobacco Products Promoted to Reduce Harm; PI: Peter Shields, M.D.). National Cancer Institute, Washington, DC. Nonsmoker (N = 5) and smoker subjects (N = 3) were between the ages of 18 and 65. Smokers smoked at least 10 cigarettes daily for the past year had exhaled carbon monoxide (CO) levels of > 10 ppm. The nonsmokers have smoked less than 100 cigarettes in their life time. Smoking status was confirmed through CO measures and self-reported tobacco use diaries. All subjects were in good physical and mental health. Fasting blood was collected through venipuncture at various time points during the study. Samples were stored at – 80 °C until further analysis.

DNA isolation

DNA from control and EB-treated human fibrosarcoma (HT1080) cells was isolated using standard phenol-chloroform extraction [35]. DNA concentrations were estimated by nanodrop UV spectrophotometer (Thermo Scientific, Waltham, MA), and the DNA purity was assessed from A₂₆₀/A₂₈₀ absorbance ratios, which was typically between 1.8 and 1.9. DNA amounts were accurately determined by dG quantitation in enzymatic hydrolysates as described previously [35, 36].

DNA from human blood samples was isolated using the manufacturer's protocol for DNA purification from buffy coat (Qiagen, Valencia, CA) [37] with minor modifications [36]. DNA isolation from rat liver was performed using the manufacturer's protocol for DNA purification from tissue (Qiagen, Valencia, CA). Briefly, 100–200 mg of liver tissue was

homogenized in 4 mL of cell lysis buffer. Cell lysis was performed overnight following addition of 15 μ L of proteinase K solution (20 mg/mL). On the following day, RNA digestion was performed by addition of 15 μ L of RNase A solution (4 mg/mL) and incubation at room temperature for 2 h. Protein precipitation solution (1 mL) was added to the cell lysate, and the mixture was vortexed at a high speed for 20 s and centrifuged at 2000g for 15 min for removal of proteins. DNA was precipitated with isopropyl alcohol (5 mL) and washed twice with 1 mL of 70% ethanol in water.

Sample preparation and EB-GII enrichment (Scheme 2)

DNA samples (3–76 μ g in water) were spiked with 3 fmol of $^{15}\text{N}_5$ -EB-GII internal standard and heated at 90 °C for 0.5 h to release EB-GII adducts from the DNA backbone as free bases. Partially depurinated DNA was removed by ultrafiltration with Nanosep 10K filters (Pall Life Sciences, Ann Arbor, MI) at 5000 g for 10 min. The filtrates containing EB-GII and its internal standard were subjected to offline HPLC purification using an Agilent 1100 series HPLC equipped with a UV detector and an automated fraction collector (Agilent Technologies, Palo Alto, CA). Offline HPLC purification was carried out using Zorbax Eclipse XDB-C18 column (4.6 \times 150 mm, 5 μ m, from Agilent Technologies, Palo Alto, CA) eluted at flow rate of 1 mL/min with a gradient of 0.4% formic acid in Milli-Q water (A) and HPLC grade acetonitrile (B). UV absorbance was monitored at 254 nm. Solvent composition was maintained at 0% for 5 min and then linearly changed to 3% in 10 min and further to 40% B in 5 min. The solvent composition was returned to 0% acetonitrile in 5 min and held at 0% for 15 min for column equilibration. Either 2'-deoxyadenosine (dA, 0.60 nmol) or 2'-deoxythymidine (dT, 0.62 nmol on column) was added as retention time markers, which eluted at 13.2 or 18.3 min, respectively. EB-GII typically eluted at 16.4 min. HPLC fractions containing EB-GII and its internal standard (15.8 – 17.8 min, 1 mL each) were collected in 1.2 mL MS total recovery vials (Thermo Fischer Scientific, Waltham, MA), concentrated under vacuum, and re-dissolved in water (10 μ L) for nano-HPLC-nanoESI⁺-HRMS³ analysis. Typical injection volume was 5 μ L.

nanoLC/ESI⁺-HRMS³ analysis of EB –GII

A Nano2D-LC HPLC system (Eksigent, Dublin, CA) with 5 μ L injection loop was interfaced to an LTQ Orbitrap Velos instrument equipped with a nanospray source (Thermo Fisher Scientific Corp., Waltham, MA). HPLC solvents were LC-MS grade water containing 0.01% acetic acid (A) and LC-MS grade acetonitrile containing 0.02% acetic acid (B). Samples (5 μ L) were injected onto a trapping column (Symmetry C18 nanoAcquity, 0.18 \times 20 mm, Waters Corp., Millford, MA) in line with a nano-LC column (0.075 \times 200 mm). The nano-LC column was prepared by manually packing a fused-silica emitter (New Objective, Woburn MA) with Synergi Hydro-RP, 80Å, 4 μ m chromatographic packing (Phenomenex, Torrance, CA). Following sample injection (5 μ L), the HPLC flow (2% B) was maintained at 1 μ L/min for 5.5 min to enable sample loading onto the trapping column. The HPLC flow was then decreased to 300 nL/min and maintained at 2% B for 0.5 min. The organic phase content linearly increased to 25% B in 19 min and further to 50% B in 10 min, returned to 2% B in 2 min, and equilibrated for 7 min. Under these conditions, EB-GII eluted as a sharp peak at 20 min.

HPLC-MS³ analyses were conducted in the nanoESI⁺ mode using an LTQ Orbitrap Velos instrument (Thermo Scientific, Waltham, MA). Typical instrument settings included the spray voltage at 2.0 kV, capillary temperature at 350 °C, and S-Lens RF level at 40%. MS analysis was performed by fragmenting [M + H]⁺ ions of EB-GII (*m/z* 222.1) via collision induced dissociation (CID) in the linear ion trap, with a normalized collision energy set at 25 units and the isolation width of 1.0 amu. The resulting MS/MS fragment ions at *m/z* 152.1 corresponding to protonated guanine [Gua + H]⁺ were subjected to further fragmentation in the high collision dissociation (HCD) cell using nitrogen as collision gas, normalized collision energy of 75 units, and an isolation width of 1.0 amu. The resulting MS³ fragment ions were detected in the mass range of *m/z* 50 to *m/z* 270 using the Orbitrap mass analyzer at a resolution of ~ 25,000. EB-GII was quantified using extracted ion chromatograms from the combined signal of *m/z* 135.0301 ([Gua – NH₃]⁺) and *m/z* 153.0407 ([Gua – NH₃ + H₂O]⁺) at a mass tolerance of 5 ppm. The MS³ fragments at *m/z* 153.0407 ([Gua – NH₃ + H₂O]⁺) correspond to the adduction of water to *m/z* 135.0301 ([Gua – NH₃]⁺) in the collision cell [38]. The ¹⁵N₅ labeled internal standard ([¹⁵N₅]-EB-GII) was quantified using an analogous MS³ scan event consisting of fragmentation of *m/z* 227.1 ([M + H]⁺) to *m/z* 157.1 [¹⁵N₅-Gua + H]⁺ and further to *m/z* 139.0183 ([¹⁵N₅-Gua – NH₃]⁺). Extracted ion chromatograms corresponding to the sum of *m/z* 139.0183 ([¹⁵N₅-Gua – NH₃]⁺) and *m/z* 157.0288 ([¹⁵N₅-Gua – NH₃ + H₂O]⁺) at 5 ppm were generated and used for quantitation (Figure 1). A full scan event was also performed over the mass range of *m/z* 100–500 at a resolution of 7500 to monitor for any co-eluting matrix components. EB-GII amounts in DNA were expressed as adduct numbers per 10⁸ normal nucleotides.

NanoLC/ESI⁺-HRMS³ standard curves were constructed by analyzing aqueous solutions containing fixed amounts of ¹⁵N₅-EB-GII (5 fmol) and increasing amounts of EB-GII (0.1, 0.5, 1, 3, 5, and 10.0 fmol) (in triplicate), followed by regression analysis of the actual and the observed amounts of EB-GII (Figure S-2). 5 μL injections were made out of a 25 μL sample volume. Solvent blanks were periodically injected to monitor for any potential analyte carry-over.

Method validation

DNA isolated from unexposed human leukocyte DNA (150 μg aliquots, in triplicate) was spiked with fixed amount of ¹⁵N₅-EB-GII (3 fmol, internal standard) and increasing amounts of EB-GII (0, 0.2, 0.5, 1, 3, 5, 7 or 10 fmol). Samples were processed by neutral thermal hydrolysis, ultrafiltration, and off-line HPLC as described above and subjected to nanoLC/ESI⁺-HRMS³ analysis. The observed amounts of EB-GII were plotted against the theoretical values, followed by regression analysis (Figure 2).

Determination of method LOD, LOQ, precision, and accuracy

The LOD values of the quantitative method for EB-GII were determined by spiking blank human DNA (150 μg) with increasing amounts of EB-GII (0, 0.05, 0.1, or 0.2 fmol) and a fixed amount of ¹⁵N₅-EB-GII (3 fmol), followed by sample processing and nanoLC/ESI⁺-HRMS³ analysis by standard methodology. The LOD value was determined as the analyte amount that consistently produced signal-to-noise ratios above 3. The limit of quantitation

was defined as minimum analyte amount that produced a coefficient of variation less than 15% and a signal-to-noise ratio (S/N) greater than 10.

To evaluate the inter-day and intra-day accuracy and precision of the new method, EB-GII (0.2 fmol) and $^{15}\text{N}_5$ -EB-GII (3.0 fmol) were spiked into blank human DNA (150 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 EB-GII and A_a is the actual analyte amount added.

Results and Discussion

Because of their critical role in cancer development, DNA adducts represent mechanism-based biomarkers of carcinogen exposure, and their quantitation is particularly useful for cancer risk assessment. DNA adducts are also valuable in mechanistic studies linking tumorigenic effects of environmental and industrial carcinogens to specific electrophilic species generated from their metabolism. As discussed above, BD is among the most important industrial and environmental chemicals classified as known carcinogens [39, 40]. Humans can be exposed to BD in an occupational setting at BD monomer/polymer industries [11] or as a result of inhaling cigarette smoke [9], automobile exhaust [8], and smoke from cooking and wood fires [10]. This is a cause of concern because of the established carcinogenicity of BD [4–7].

The long term goal of this work is to develop sensitive biomarkers of human exposure to BD. We recently reported an isotope dilution accurate mass spectrometric method for another BD-DNA adduct, N7-(2,3,4-trihydroxybut-1-yl)-guanine (N7-THBG), which was detected in leukocyte DNA of smokers, nonsmokers, and occupationally exposed workers [36]. Unexpectedly, N7-THBG was also observed in samples from individuals with no known exposure to BD, and no significant decrease in N7-THBG levels was observed upon smoking cessation [36]. Therefore, THBG may be formed endogenously and does not accurately reflect human exposure to BD.

In an effort to develop a new BD-specific DNA biomarker, we turned our attention to structurally analogous EB-G adducts (Scheme 1). EB-GI and EB-GII are formed upon N7-guanine alkylation by 3,4-epoxybut-1-ene (EB). Because EB is formed in lower amounts than 3,4-epoxy-1,2-butanediol (EBD), *in vivo* concentrations EB-GI and EB-GII adducts are approximately 10-fold lower than those of THBG following exposure to BD [41], calling for a highly sensitive methodology.

Our method for quantitative analysis of EB-G adducts *in vivo* starts with DNA extraction from cells or tissues (Scheme 2). DNA purity is estimated by UV spectrophotometry (A₂₆₀/A₂₈₀ ratios) to minimize any contamination with cellular proteins. DNA samples (7–76 μg) are spiked with isotopically labeled internal standard ($^{15}\text{N}_5$ -EB-GII) to account for any analyte loss during sample workup and to allow for absolute quantification. Since N7-guanine alkylation generates a positive charge on the adducted base and destabilizes the N-glycosidic bond, EB-G adducts can be selectively released from the DNA backbone as free bases by neutral thermal hydrolysis (Scheme 2) [41, 42]. This step was optimized upon

analyzing EB-treated calf thymus DNA. A range of depurination conditions were tested, including heating at 90 °C for 0.5 h (pH 6.8), 70 °C for 1 h (pH 6.8), and acidic thermal hydrolysis at 90 °C for 0.5 h (0.1N HCl). Since all three conditions were equally effective at releasing EB-G adducts from the DNA backbone (not shown), neutral thermal hydrolysis at 90 °C for 0.5 h in water was selected to minimize the depurination of unmodified nucleobases. Following ultrafiltration to remove partially depurinated DNA, EB-GII adducts were enriched by offline HPLC. Retention time markers (dT or dA) were added to account for any sample-dependent retention time shifts. Under our HPLC conditions, dA, EB-GI, EB-GII, and dT typically eluted at 13.2, 15.3, 16.4, and 18.3 min, respectively. In our experience, offline HPLC purification is the best option for isolating trace amounts of DNA adducts from DNA hydrolysates prior to LC-MS analysis because it affords nearly quantitative analyte recovery, can be readily automated, and avoids the introduction of solid phase particles which can clog nano HPLC columns [43]. However, cross-contamination and analyte carryover can be a serious problem, so multiple HPLC blanks must be introduced throughout the analysis.

NanoLC/ESI⁺-HRMS³ method development

Our ultimate goal was to develop a quantitative method sensitive enough to detect EB-G adducts in tissues of laboratory animals exposed to sub-ppm levels of BD and in leukocyte DNA of smokers and occupationally exposed individuals. NanoHPLC-nanospray HPLC-MS methodology was employed because of its enhanced sensitivity as compared to conventional capillary LC-MS. Nanospray ionization is more efficient than conventional electrospray because of an improved ionization efficiency and an increased ion transport from the source into the mass spectrometer [44]. A number of stationary HPLC phases were tested, including Zorbax SB-C18 (Agilent Technologies), Hypercarb (Phenomenex), Luna C18 (Phenomenex), Synergi Hydro- RP 80Å (Phenomenex), and Synergi Polar-RP (Phenomenex). These solid phases were manually packed into commercial fused-silica Self-Pack PicoFrit emitters (200 mm x 75 µm, New Objective), with a 15 µm tip orifice diameter. The best results in terms of analyte retention and HPLC peak shape were obtained when using a Synergi Hydro-RP column eluted with a gradient of 0.01% acetic acid in water (A) and 0.02% acetic acid in acetonitrile (B) (Figure 1) This system has afforded a good EB-GII peak shape (Peak Asymmetry factor=1.16, tailing factor = 1.08), along with good retention time (RT 20.7 min) and theoretical plates (N) close to 40000, without any co-eluting peaks (Figure 1).

Our initial method development efforts have attempted to employ nanoHPLC-ESI-MS/MS on a triple quadrupole mass spectrometer. However, preliminary experiments with EB-G I and EB-G II pure standards spiked into synthetic DNA indicated that the sensitivity of this approach was insufficient for BD-DNA adduct detection in human samples (Figure S-1A). Other published HPLC-ESI-MS/MS methods for EB-guanine adducts [41] similarly reported moderate sensitivity for this analyte (typical LOD, 0.5 adducts/10⁶nucleotides). To achieve detection limits in the low fmol to amol range (1 per 10⁸–1 per 10⁹ nucleotides) as required for our *in vivo* studies, we employed nanoLC/ESI⁺-HRMS³ methodology on an Orbitrap Velos mass spectrometer. We and others have recently reported the use of high resolution mass spectrometry for DNA adduct analysis in complex samples and have shown

that it dramatically reduces the matrix background, leading to greatly improved signal to noise ratios [37, 45, 46].

An LTQ Orbitrap Velos mass spectrometer was operated in the high resolution MS³ scan mode to allow for highly selective detection of EB-GII adducts in a complex biological matrix. Protonated molecules of the analyte were trapped and fragmented in the linear ion trap (LTQ), and specific MS² fragments were axially ejected, fragmented in the HCD cell, and collected in a C-shaped ion trap (C-Trap) to be analyzed in the Orbitrap for accurate mass analysis. Initial fragmentation of protonated molecules of EB-GII and ¹⁵N₅-EB-GII (m/z 222.1 [M + H]⁺ → m/z 152.1 [Gua + H]⁺ and m/z 227.1 [¹⁵N₅-M + H]⁺ → m/z 157.1 [¹⁵N₅-Gua + H]⁺) was performed in the linear ion trap via CID, while subsequent fragmentation (m/z 152.1 [Gua + H]⁺ → m/z 135.0301 [Gua – NH₃]⁺, 153.0407 [Gua – NH₃ + H₂O]⁺) and m/z 157.1 [¹⁵N₅-Gua + H]⁺ → m/z 139.0183 ([¹⁵N₅-Gua – NH₃]⁺), 157.0288 (¹⁵N₅-Gua – ¹⁵NH₃ + H₂O) was performed in the HCD cell (Scheme 3). The ions at 153.0407 [Gua – NH₃ + H₂O]⁺ are generated via neutral gain of H₂O by [Gua – NH₃]⁺ ions (Scheme 3), a process that has been shown to take place in the MS collision cell due to the presence of residual water [38]. MS³ fragment ions were analyzed in the accurate mass mode over a range m/z 50–270 at a resolution of ~ 25,000. We found that the MS³ scan mode afforded better signal to noise ratios as compared to the MS² due to its selectivity for the analyte of interest in the presence of co-eluting contaminant peaks (Figure S-1B). Using nanoLC/ESI⁺-HRMS³ methodology, the sensitivity for EB-G adducts was dramatically improved as compared to standard SRM on a triple quadrupole mass analyzer (Compare Figures S-1A and S-1B), with excellent S/N ratios for the low and sub fmol analyte amounts. As an additional benefit, MS³ spectra are available for each sample, providing additional confirmation of analyte identity. Quantitation is based on peak areas corresponding to the analyte and its internal standard. Standard curves obtained by nanoLC/ESI⁺-HRMS³ analysis of known amounts of pure standards of EB-G and its ¹⁵N₅-labeled internal standard are given in the supplementary Figure S-2.

Our initial studies have attempted to quantify both regioisomers of the adduct, EB-GI and EB-GII (Scheme 1). Under our offline HPLC conditions, pure standards of EB-GI and EB-GII eluted as sharp peaks well resolved from each other (not shown). However, when the two analytes were spiked in synthetic oligodeoxynucleotide or into blank human DNA (150 µg), processed as described above, and subjected to nanoLC/ESI⁺-HRMS-SRM analysis, a co-eluting peak was observed at similar HPLC retention time as EB-GI (Figure S-3). Our efforts to separate EB-GI from this interfering impurity under various chromatographic conditions (a range of HPLC stationary phases, HPLC gradients, and HPLC buffers such as 0.05% formic acid, 5 mM ammonium acetate buffer (pH 6.8) and 0.1% formic acid with a gradient of methanol or acetonitrile as organic phase) were not successful. Although the co-elute signal was somewhat decreased when narrowing the precursor ion (m/z 222.1) isolation width from 3.0 amu to 1.0 amu, this also led to a significant loss of sensitivity for the analyte (not shown). MS² experiments have revealed that the co-eluting impurity from sample matrix had a different MS² fragmentation pattern as compared to authentic EB-GI, but unfortunately shared the same major fragment ion at m/z 152.0567 (Figure S3). Our efforts to eliminate the interfering peak using MS³ also proved unsuccessful and the co-eluting impurity interfered with accurate analysis of EB-GI (results not shown). We

therefore focused our efforts on EB-GII (Scheme 1), which is formed upon nucleophilic attack of N7-guanine at an internal carbon of EB and is produced in similar amounts as its regioisomer [41].

NanoLC/ESI⁺-HRMS³ Method Validation

The newly developed method was validated by analyzing samples prepared by spiking known amounts of EB-GII (0.2 – 10 fmol) and 3 fmol of ¹⁵N₅-EB-GII internal standard into 150 µg of blank human buffy coat DNA. Samples were analyzed by the methodology described above. An excellent correlation ($R^2 = 0.9953$) was observed between the spiked and the observed amounts of EB-GII in the targeted sample matrix analysis (Figure 2). Method accuracy and interday/intraday precision were determined by analyzing replicate samples of EB-GII (0.2 fmol) spiked into blank human DNA (see example in Figure S-4). The accuracy of the analytical method for 0.2 fmol spiked into 150 µg of blank human DNA was calculated as $92.9 \pm 7.19\%$ ($N = 9$), while the interday and intraday precision were less than 8% RSD (Table 1). In order to evaluate the method's sensitivity (LOD), aliquots of blank human DNA (150 µg each) were spiked with known amounts of EB-GII standard (0, 0.05, 0.1 or 0.2 fmol), followed by thermal hydrolysis and sample processing as described above (Scheme 2). The nanoLC/ESI⁺-HRMS³ limit of detection for EB-GII was determined as 0.05 fmol in 150 µg of DNA (0.1 adducts/10⁹ nucleotides), which gave the signal-to-noise ratio 3. No EB-GII was detected in blank human DNA derived from blood leukocytes of a nonsmoker, confirming that there was no artifactual formation of EB-GII during the sample preparation and analysis. The method's limit of quantitation was defined as the lowest amount of EB-GII spiked into blank human DNA (150 µg) that afforded the signal-to-noise ratios of >10 and intra/inter day precision within 15% CV. We found that the LOQ value of our LC-MS³ method for EB-guanine II was 0.2 fmol analyte in 150 µg of DNA, corresponding to 0.4 adducts/10⁹ nucleosides (see Figure S-4).

EB-GII quantitation in Human Cell Culture

The applicability of the new HPLC-MS³ analytical method for EB-GII was initially tested by quantifying these adducts in human fibrosarcoma (HT1080) cells treated with increasing concentrations of EB (0.5–10 µM) for 3 h. These concentrations are well below the EC₅₀ of EB in HT1080 cells (13.8 mM). Following DNA extraction, EB-GII adducts were released by neutral thermal hydrolysis and analyzed by nanoLC/ESI⁺-HRMS³ as described above. Adduct concentrations in cells treated with 0.5 µM – 10 µM DEB increased in a concentration-dependent manner from 1.15 ± 0.23 to 10.11 ± 0.45 adducts/10⁸ nucleotides (Figure 3).

EB-GII quantitation in rat liver tissue DNA

The new method was next used to quantify EB-GII DNA adducts in liver tissue DNA of F344 rats exposed to sub ppm concentrations of BD (0.5 – 1.5 ppm). According to literature reports, laboratory rats are a better animal model of human exposure to BD than laboratory mice due to interspecies similarities in the metabolic pathways [47]. We chose low to sub-ppm exposures because this concentration range is comparable to occupational BD exposures in polymer or monomer industries [11]. NanoLC/ESI⁺-HRMS³ analyses of EB-GII in liver DNA of BD-exposed rats have revealed prominent analyte peaks with little to no

background noise (Figure 4A). A dose dependent increase in adduct amounts was observed in animals treated with higher BD concentrations, with EB-GII numbers changing from 0.17 ± 0.05 adducts/ 10^8 nucleotides following 0.5 ppm exposure to 0.33 ± 0.08 adducts/ 10^8 nucleotides following 1.0 ppm exposure, and further to 0.50 ± 0.04 adducts/ 10^8 nucleotides following exposure to 1.5 ppm BD (Figure 4B). No EB-GII adducts were detected in control animals exposed to ambient air only.

The new method was further applied to determine the *in vivo* half-lives of EB-GII adducts in liver of laboratory rats exposed to 1250 ppm BD. Only 7 μg of DNA was used in this case due to the high adduct levels in these samples. EB-GII adduct concentrations in rat tissues gradually decreased 1, 3 and 6 days post exposure to BD (Figure 5A). Data processing via first order kinetics analysis yielded the EB-GII half-life in rat liver DNA as 2.20 ± 0.12 days (Figure 5B), which is significantly shorter than the half-life of structurally analogous N7-THBG adducts *in vivo* ($t_{1/2} = 3.6\text{--}4.0$ days) [16].

Attempted EB –GII quantitation in human blood leukocyte DNA

To test the ability of the new method to detect BD-DNA adducts in humans, it was applied to blood leukocyte DNA of confirmed smokers ($N = 8$). Smokers are exposed to BD due to its relatively high concentrations in cigarette smoke (8.5–48.2 $\mu\text{g}/\text{cigarette}$) [9]. Although we were able to successfully detect EB-GII adducts in human DNA (Figure S-5), adduct amounts were below the limit of quantitation of our method (0.4 adducts/ 10^9 nucleotides). Unfortunately, DNA amounts could not be increased due to the limited human sample availability. Furthermore, our *in vivo* persistence data (Figure 5) suggest that EB-GII adducts are rapidly released from the DNA backbone by spontaneous hydrolysis and/or active repair. Our current efforts are to develop a nanoLC/ESI⁺-HRMS³ methodology for EB-GII adducts in human urine, which is readily available, but will require additional sample cleanup steps to remove salts and other polar matrix compounds prior to analysis. Our final goal is to develop analytical methodology that has adequate sensitivity, accuracy, and precision for quantitation of EB-G II adducts in human populations.

Conclusions

In conclusion, sensitive, accurate, and specific nanoLC/ESI⁺-HRMS³ isotope dilution methodology has been developed for accurate and precise quantitation of BD-specific EB-GII adducts *in vivo*. The applicability of this method was demonstrated by quantitation of EB-GII adducts in human fibrosarcoma cells exposed to low micromolar concentrations of EB and in liver DNA of laboratory rats exposed to sub-ppm concentration of BD, which mimic occupational exposure in humans. The method had adequate sensitivity to quantify EB-GII DNA adducts in human leukocyte DNA, provided that that adequate DNA amounts are available for analysis. We conclude that nanoLC/ESI⁺-HRMS³ methodology holds a great promise in quantitative analyses of trace levels of DNA adducts in human and animal samples due to an increased sensitivity, specificity, and detailed structural confirmation from MS³ spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BD

1,3-butadiene

EB

3,4-epoxy-1-butene

EBD

1,2-dihydroxy-3,4-epoxybutane

DEB

1,2,3,4-diepoxybutane

EB-GI

N-7-(2-hydroxy-3-buten-1-yl) guanine

EB-GII

N-7-(1-hydroxy-3-buten-2-yl) guanine

N7-THBG

N7-(2,3,4-trihydroxybut-1-yl) guanine

nanoHPLC-nanoESI⁺-HRMS³

nanoflow high performance liquid chromatography nano electrospray ionization high resolution tandem mass spectrometry in positive mode

SPE

solid-phase extraction

LOD

limit of detection

LOQ

limit of quantitation

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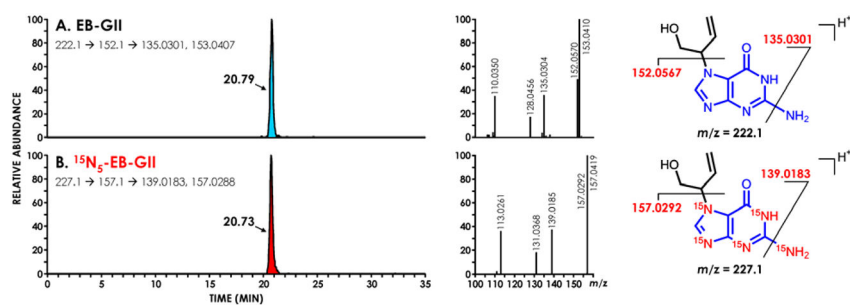


Figure 1. Nano-HPLC-nanoESI⁺-HRMS³ extracted ion chromatograms and MS³ spectra of synthetic EB guanine II (0.1 fmol) (A) and [¹⁵N₅]-EB guanine II (5 fmol) (B).

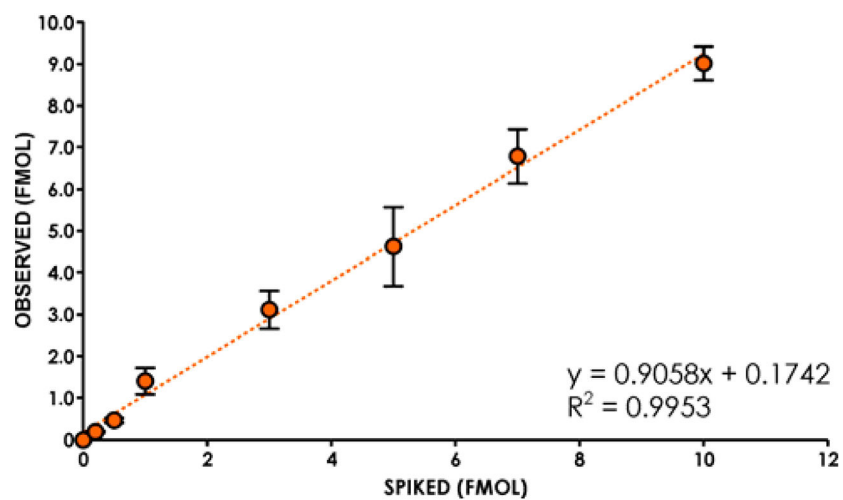


Figure 2. Nano-HPLC-nanoESI⁺-HRMS³ method validation: correlation between the spiked and the observed amounts of EB-GII spiked into blank DNA. DNA isolated from nonsmoker blood leukocytes (150 μ g) was spiked with 0, 0.2, 0.5, 1, 3, 5, 7 or 10 fmol of EB-GII and 3 fmol of ¹⁵N₅- EB-GII (internal standard), followed by sample processing and nano-HPLC-nanoESI⁺-HRMS³ analysis.

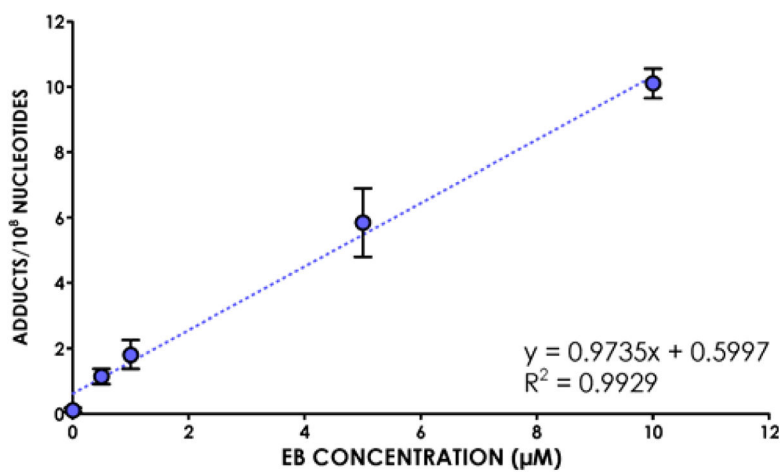


Figure 3. Concentration-dependent formation of EB-GII adducts in HT1080 cells treated with increasing amounts of EB (0.5–10 µM).

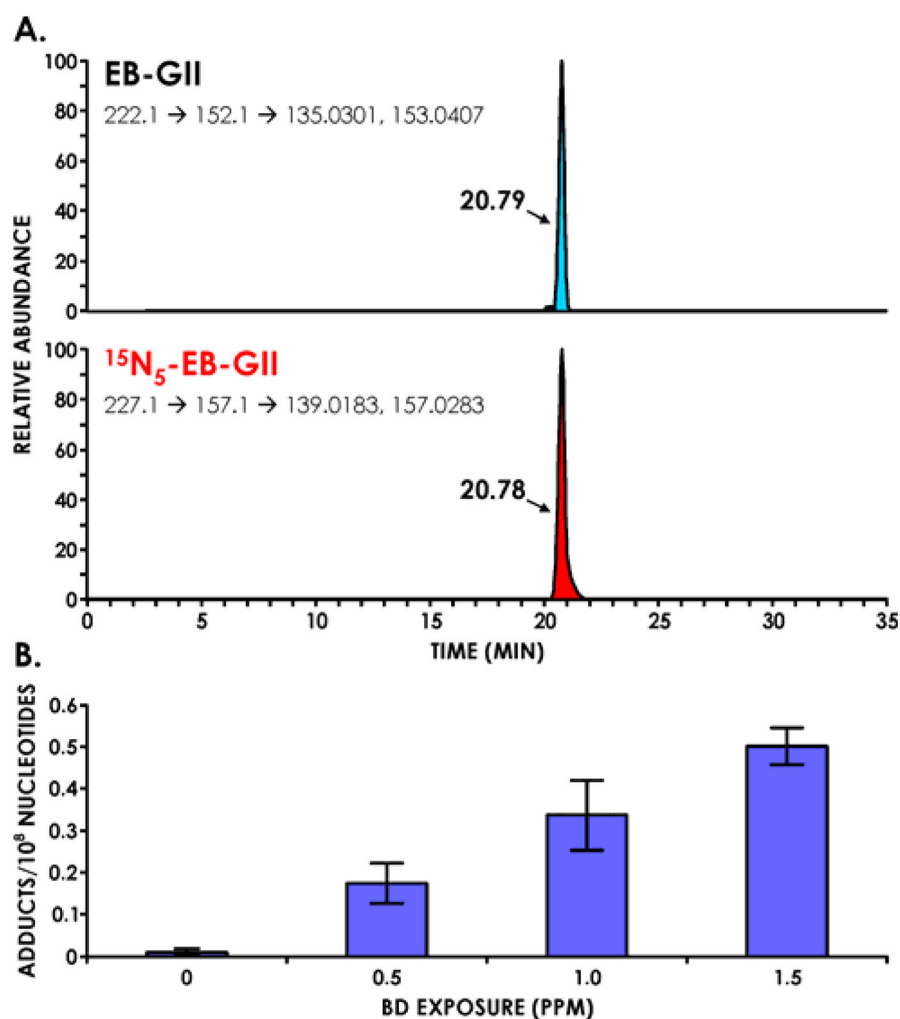


Figure 4.
A. Representative extracted ion chromatogram for nano-HPLC-nanoESI⁺-HRMS³ analysis of EB-GII adducts in liver DNA of a laboratory rat exposed to 1.0 ppm BD by inhalation for 2 weeks. Liver DNA (53.3 μ g) was spiked with ¹⁵N₅-EB-GII (internal standard for quantitation) and subjected to neutral thermal hydrolysis, sample processing, and nano-HPLC-nanoESI⁺-HRMS³ analysis on an Orbitrap Velos mass spectrometer. **B.** Concentration-dependent formation of EB-GII adducts in liver DNA of laboratory rats exposed to low ppm (0.5, 1.0, 1.5 ppm) concentrations of BD.

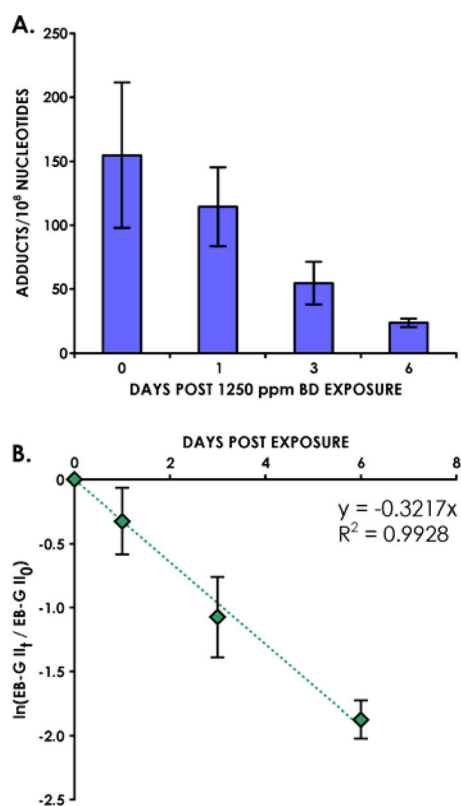
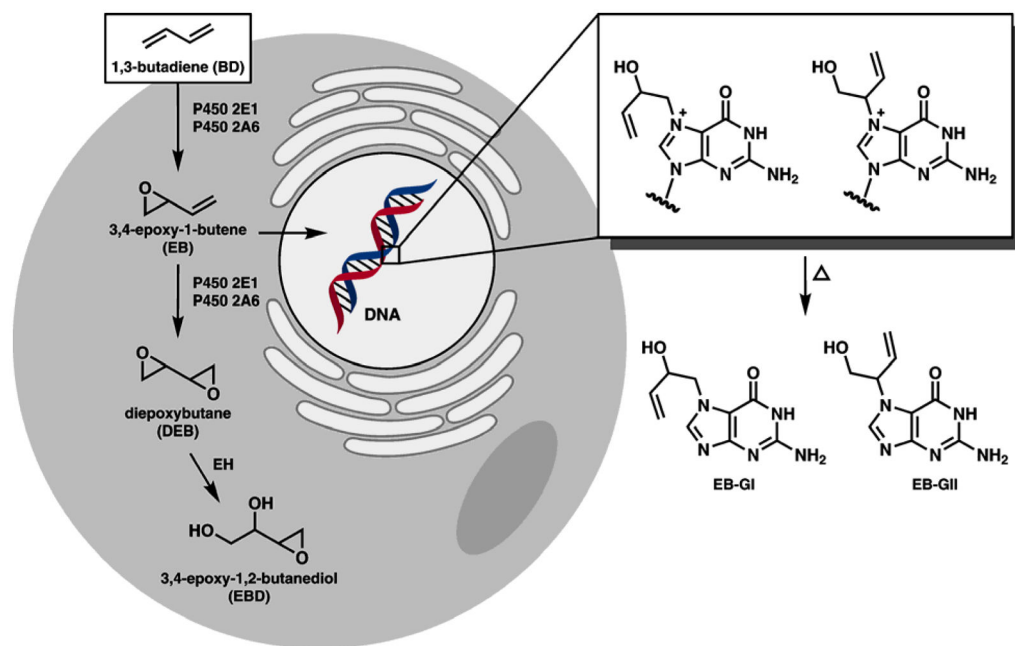
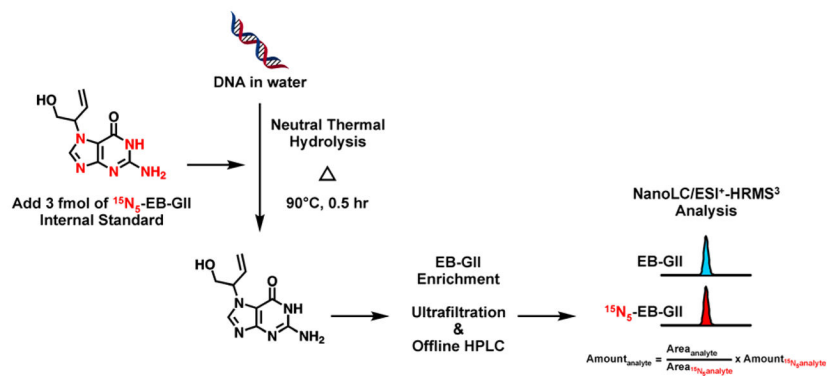


Figure 5. Persistence of EB-GII adducts in liver DNA of rats exposed to 1250 ppm BD by inhalation (A) and first order kinetic analysis for estimation of adduct half-life *in vivo* (B).

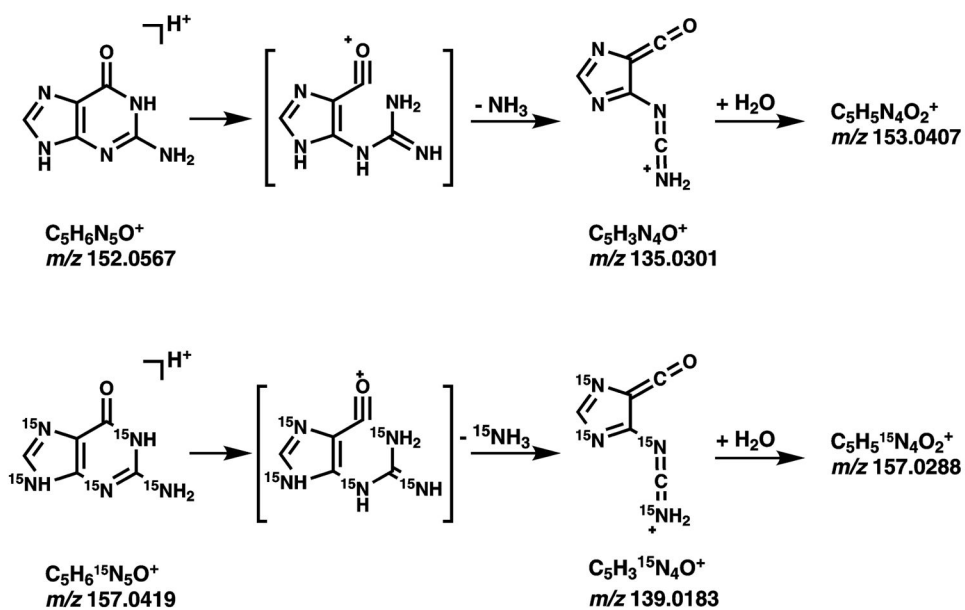


Scheme 1.

Bioactivation of BD to electrophilic epoxides and the formation of EB-guanine adducts in DNA. EH, epoxide hydrolase; EB-GI, N-7-(2-hydroxy-3-buten-1-yl) guanine; EB-GII, N-7-(1-hydroxy-3-buten-2-yl) guanine.



Scheme 2.
Analytical procedure for isotope dilution nanoLC/ESI⁺ HRMS³ analysis of EB-GII in DNA.

**Scheme 3.**

Dissociation scheme to explain the formation of fragment ions at 153.0407 [Gua - NH₃ + H₂O]⁺. Adopted from [38].

Table 1

Accuracy and precision results for NanoLC/ESI⁺ HRMS³ analysis of EB-GII (0.2 fmol) spiked into 150 µg of blank human DNA.

Day 1	Mean	0.17
	RSD (%)	3.75
	Accuracy(%)	86.0
	N	3
Day 2	Mean	0.18
	RSD (%)	1.23
	Accuracy(%)	91.8
	N	3
Day 3	Mean	0.20
	RSD (%)	5.29
	Accuracy(%)	100.8
	N	3
Interday	Mean	0.18
	RSD (%)	7.74
	Accuracy(%)	92.9
	N	9