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Isotope dilution nanoLC/ESI⁺-HRMS³ quantitation of urinary N7-(1-hydroxy-3-buten-2-yl) guanine adducts in humans and their use as biomarkers of exposure to 1, 3-butadiene

Dewakar Sangaraju[†], Emily J. Boldry[†], Yesha M. Patel[‡], Vernon Walker[§], Irina Stepanov[¶], Daniel Stram[‡], Dorothy Hatsukami[£], and Natalia Tretyakova^{†,*}

[†]Department of Medicinal Chemistry and Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

[‡]Division of Biostatistics, Keck School of Medicine and Children's Cancer Group, University of Southern California, Los Angeles, CA 90089

[§]Department of Pathology and Laboratory Medicine, University of Vermont, Burlington, VT 05405

[¶]School of Public Health, Division of Environmental Health Sciences and Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

[£]Department of Psychiatry and Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

Abstract

1,3-Butadiene (BD) is an important industrial and environmental chemical classified as a known human carcinogen. Occupational exposure to BD in the polymer and monomer industries is associated with an increased incidence of lymphoma. BD is present in automobile exhaust, cigarette smoke, and forest fires, raising concern about potential exposure of general population to this carcinogen. Following inhalation exposure, BD is bioactivated to 3,4-epoxy-1-butene (EB). If not detoxified, EB is capable of modifying guanine and adenine bases of DNA to form nucleobase adducts, which interfere with accurate DNA replication and cause cancer-initiating mutations. We have developed a nanoLC/ESI⁺-HRMS³ methodology for N7-(1-hydroxy-3-buten-2-yl) guanine (EB-GII) adducts in human urine (limit of detection: 0.25 fmol/mL urine, limit of quantitation: 1.0 fmol/mL urine). The new method was successfully used to quantify EB-GII in urine of F344 rats treated with 0 – 200 ppm BD, occupationally exposed workers, and smokers belonging to two different ethnic groups. EB-GII amounts increased in a dose-dependent manner in urine of laboratory rats exposed to 0, 62.5, or 200 ppm BD. Urinary EB-GII levels were significantly increased in workers occupationally exposed to 0.1–2.2 ppm BD (1.25 ± 0.51 pg/mg creatinine) as compared to administrative controls exposed to < 0.01 ppm BD (0.22 ± 0.08 and pg/mg creatinine) ($p = 0.0024$), validating the use of EB-GII as a biomarker of human exposure to BD. EB-GII was

*To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, 2231 6th Street SE - 2-147 CCRB, Minneapolis, MN 55455, USA. phone: (612) 626-3432 fax: (612) 624-3869, trety001@umn.edu.

Notes

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also detected in smokers' urine, with European American smokers excreting significantly higher amounts of EB-GII than African American smokers (0.48 ± 0.09 vs 0.12 ± 0.02 pg/mg creatinine, $p = 3.1 \times 10^{-7}$). Interestingly, small amounts of EB-GII were observed in animals and humans with no known exposure to BD, providing preliminary evidence for its endogenous formation. Comparison of EB-GII adduct levels in urine to urinary mercapturic acids of BD (MHBMA, DHBMA) and genetic polymorphisms were also attempted in the genotyped smoker ethnic groups cohort.

TOC image

Keywords

urinary DNA adducts; 1,3-butadiene; smoking; occupational exposure; LC-MS/MS

Introduction

1,3-butadiene (BD) is a carcinogenic gas found in cigarette smoke, automobile emissions, and in wood smoke from forest fires.¹ Human exposure to BD can also occur in occupational settings such as styrene-butadiene polymer industries and synthetic rubber production facilities, leading to an increased risk of lymphoma.^{2,3} BD is produced in cigarette smoke by a thermal cracking process due to the combustion of carbohydrates, amino acids, phytosterols, paraffins, and other tobacco components.⁴⁻⁶ BD undergoes cytochrome P450 2E1-mediated metabolic activation to 3,4-epoxy-1-butene (EB) (Scheme 1).^{7,8} EB is mutagenic in cultured human lymphoblasts⁹ and is thought to contribute to carcinogenicity of BD in laboratory animals with observed species differences in metabolic activation of BD to its reactive epoxides.^{10,11}

If not inactivated via metabolic hydrolysis and/or conjugation with glutathione, EB can enter the cell nuclei and reacts with the N7 position of guanine bases in DNA to form regioisomeric N7-(2-hydroxy-3-buten-1-yl) guanine (EB-GI) and N7-(1-hydroxy-3-buten-2-yl) guanine (EB-GII) adducts (Scheme 1).^{12,13} These adducts are hydrolytically labile due to the positive charge generated upon alkylation of the N7 position of guanine, resulting in their spontaneous depurination ($t_{1/2}$, 2.20 ± 0.12 days).^{14,15} EB-GI and EB-GII have been detected in EB-treated calf thymus DNA,¹⁶ in EB-treated human cells in culture,¹⁷ and in tissues of laboratory rats and mice exposed to BD by inhalation.^{12,17,18} Liver DNA of F344 rats exposed to 625 ppm BD for 6 hours/day, 5 days/week for 4 weeks contained 0.1–1.2

EB-GI and 0.2–0.9 EB-GII adducts per 10^6 guanine bases, respectively.¹⁷ Tissues of B6C3F1 mice exposed to the same BD concentrations contained higher levels of BD adducts (0.2–3.0 EB-GI and 0.1–2.4 EB-GII adducts per 10^6 guanine bases), supposedly due to more efficient bioactivation of BD to EB in this species,¹² which in turn can lead to greater sensitivity of mice towards BD-mediated cancer.¹¹

Accurate quantitation of EB-G adducts in humans exposed to BD from occupational and environmental sources is a challenging task due to their exceedingly low levels (< 1 per 10^8 guanine bases)^{19,20} and their polar nature, which leads to poor retention on common HPLC packings. Recently, we reported a quantitative isotope dilution nanoLC/ESI⁺ HRMS³ method for EB-GII adducts in human blood leukocyte DNA.¹⁹ Although this method had excellent sensitivity (limit of quantitation: 0.4 adducts/ 10^9 nucleotides), we were unable to accurately quantify EB-GII in humans DNA because of their low levels (< 0.2 adducts/ 10^9 nucleotides) and the limited availability of blood samples.

In the present work, we evaluated the possibility of quantifying EB-GII in human urine as biomarkers of exposure to BD and its bioactivation to DNA-reactive epoxides. Urine is readily available from many epidemiological studies as it can be collected in a non-invasive manner and has fewer biohazard concerns. We have developed an isotope dilution nanoLC-high resolution tandem mass spectrometry (nanoLC/ESI⁺-HRMS³) method that utilizes an Orbitrap Velos mass spectrometer for sensitive and specific quantitation of EB-GII in humans. We focused on accurate quantitation of one of the EB-G adduct regioisomers, EB-GII, because of its relative ease in quantitation without any MS interference or co-elution problems. Following validation, the new methodology was used to quantify EB-GII in the urine from laboratory rats exposed to BD by inhalation, occupationally exposed workers, and smokers of African American and European American ethnicity.

Experimental

Note: *EB is a known carcinogen and should be handled with adequate safety precautions.*

Materials

LC-MS grade water, methanol, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO). Strata X polymeric reversed phase SPE cartridges (30 mg/1 mL) were from Phenomenex (Torrance, CA). EB-GII and $^{15}\text{N}_5$ -EB-GII standards were prepared as previously reported.^{16,21} Molar concentrations of EB-GII and $^{15}\text{N}_5$ -EB-GII in standard solutions were determined by UV spectrophotometry using published molar extinction coefficient (ϵ) of $8030 \text{ M}^{-1} \text{ cm}^{-1}$ at 284 nm at neutral pH.²¹ Isotopic purity of $^{15}\text{N}_5$ -EB-GII as determined by mass spectrometry was 99.93 %. Stock solutions of EB-GII and $^{15}\text{N}_5$ -EB-GII were prepared in water and stored at -20°C . MHBMA, DHBMA, $^2\text{H}_6$ -MHBMA, and $^2\text{H}_7$ -DHBMA were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis HLB 96 well plates were procured from Waters Corp. (Milford, MA).

Animals and Inhalation Treatments

Male and female F344 rats (3 per group) were exposed to 0, 62.5, or 200 ppm BD for 2 weeks (6 hours/day, 5 days/week) using whole-body exposure chambers at the Environmental Exposure and Inhalation Health Facility at the University of Texas Medical Branch at Galveston as reported previously.²² Control rats were exposed to filtered air only. Urine was collected using metabolic chambers and stored at -80°C until analysis as described previously.²²

Human study subjects

For occupational exposure study, spot urine samples were collected from occupationally exposed human subjects in a BD production facility ($N = 23$, 30% current cigarette smokers) and control administrative workers which included both smokers and nonsmokers, ($N = 28$, 30 % current smokers).^{22–25} BD exposure was determined by personal monitoring for 8-hour work shifts on 10 separate occasions over an interval of 4 months for each study subject. The details of the occupational exposure study have been reported previously.^{23–25} This work was approved by the Institutional Review Boards at the Regional Institute of Hygiene of Central Bohemia and the University of Vermont. Archived urine samples were shipped to the University of Minnesota on dry ice and stored at -80°C until analysis.

For the study comparing smokers ($N = 29$) and nonsmokers ($N = 28$), non-smoker urine samples were from the Tobacco Research Biorepository Facility at the Masonic Cancer Center, University of Minnesota. The study was approved by the University of Minnesota Human Research Protection Programs Institutional Review Board. Nonsmoker human subjects were between 19 and 44 years old (mean age; 33 ± 12 years), smoked less than 100 cigarettes in their lifetime, and were not using any tobacco products. Smokers included in this study were a subset of subjects from the below ethnic study between the ages of 18 and 62 (Mean age: 42 ± 12 years) and smoked at least 20 ± 10 cigarettes/day, with mean carbon monoxide (CO) concentration levels of 19 ± 10 ppm. For the study of ethnic differences in smokers, 24-hour urine samples were collected from male ($N = 76$) and female ($N = 73$) smokers of African American ($N = 74$) and European American ($N = 75$) ancestry. Male (age: 42 ± 11) and female (age: 44 ± 11) subjects who smoked an average of 17 cigarettes per day for at least one year were recruited. Overall, mean CO levels in male and female smokers were 16 ± 7 and 20 ± 10 ppm, respectively. Subjects were asked to collect a 24-hour urine sample and bring it to a research clinic at the University of Minnesota. Urine samples were stored at -20°C before analysis. This study was approved by the University of Minnesota Institutional Review Board (Study # 1007M85757).

Urine sample processing and EB-GII adduct enrichment (Scheme 2)

Urine (10 μL for BD-exposed rat samples, 200 μL for human samples) was centrifuged at $10,000 \times g$ for 15 min to remove any particulate matter. The supernatants were spiked with $^{15}\text{N}_5$ -EB-GII (5 fmol, internal standard for mass spectrometry) and subjected to solid phase extraction (SPE) on Strata X cartridges (30 mg/1 mL, Phenomenex, Torrance, CA). SPE cartridges were conditioned with 2 mL of methanol, followed by 2 mL of MilliQ water. Urine samples (10 or 200 μL) were loaded onto prepared cartridges, and the cartridges were washed with 1 mL of water, followed by 1 mL of 10% methanol in water. EB-GII and its

internal standard were eluted with 60% methanol in water, dried under vacuum, and reconstituted with 200 μ L of water containing a 2'-deoxythymidine (dT) retention time marker (2.1 nmol).

Offline HPLC purification of SPE-enriched EB-GII and its internal standard was achieved on an Agilent 1100 series HPLC system equipped with a UV detector and an automated fraction collector (Agilent Technologies, Santa Clara, CA). A Zorbax Eclipse XDB-C18 column (4.6 $\times$ 150 mm, 5 μ m, Agilent Technologies, Santa Clara, CA) was 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). Solvent composition was maintained at 0% B for 5 min and then linearly increased to 3% B 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. UV absorbance was monitored at 254 nm. dT was used as a retention time marker. Under these conditions, dT eluted at $\sim$ 18.3 min, while the retention time of EB-GII was $\sim$ 16.6 min. HPLC fractions containing EB-GII and its internal standard (16.0 – 18.0 min, 1 mL each) were collected into 1.2 mL MS total recovery vials (Thermo Fischer Scientific, Waltham, MA), concentrated under vacuum, and re-dissolved in LC-MS grade water (10 μ L) for nano-HPLC-nanoESI⁺-HRMS³ analysis. The typical injection volume was 5 μ L. HPLC blanks were injected in the beginning of each sequence and after every 5–7 runs to detect any analyte carryover.

NanoLC/ESI⁺-HRMS³ analysis of urinary EB –GII

All nanoLC/ESI⁺-HRMS³ analyses were conducted on a nano2D-LC HPLC system (Eksigent LLC, Dublin, CA) fitted with a 5 μ L injection loop and interfaced to an LTQ Orbitrap Velos instrument equipped with a nanospray source (Thermo Fisher Scientific Corp., Waltham, MA). Gradient elution was achieved using LC-MS grade water containing 0.01% acetic acid (A) and LC-MS grade acetonitrile containing 0.02% acetic acid (B). Samples were loaded onto a Symmetry C18 nanoAquity trapping column (0.18 $\times$ 20 mm, Waters Corp., Milford, MA), which was connected in line with a nano-LC column (0.075 $\times$ 200 mm) manually packed with Synergi Hydro-RP 80 $\text{\AA}$ (4 μ m) chromatographic packing (Phenomenex, Torrance, CA). The initial flow rate was maintained at 2% B and 1 μ L/min for 5.5 min to enable sample loading onto the trapping column, followed by a flow rate decrease to 300 nL/min at 2% B for 0.5 min. The percentage of solvent B was linearly increased to 25% B for 9 min and further to 90% B in 10 min at a flow rate of 300 nL/min. The flow rate was increased to 1 μ L/min, and solvent B was returned to 2% in 2 min followed by 7 min column equilibration. Under these conditions, EB-GII eluted as a sharp peak at 20 min.

Tandem mass spectrometry 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 portion of the instrument using the collision energy (CE) at 25 and the isolation width (IW) of 1.0 amu. MS/MS fragment ions at m/z 152.1 $[Gua + H]^+$ were subjected to further fragmentation in the high collision dissociation (HCD) cell of the instrument using nitrogen as a collision gas (CE = 75 units, IW = 1.0 amu). The resulting MS³ fragment ions at m/z 135.0301 ($[Gua - NH_3]^+$) and m/z 153.0411 ($[Gua - NH_3 + H_2O]^+$) were detected in the mass range of m/z 50 – 270 using the Orbitrap mass analyzer (HRMS) at a resolution of 25,000. The ¹⁵N₅ labeled

internal standard ($[^{15}\text{N}_5]\text{-EB-GII}$) was detected using an analogous MS^3 scan event consisting of fragmentation of m/z 227.1 ($[\text{M} + \text{H}]^+$) to m/z 157.1 ($[^{15}\text{N}_5\text{-Gua} + \text{H}]^+$) and further to m/z 139.0183 ($[^{15}\text{N}_5\text{-Gua} - \text{NH}_3]^+$) and m/z 157.0283 ($[^{15}\text{N}_5\text{-Gua} - \text{NH}_3 + \text{H}_2\text{O}]^+$). Extracted ion chromatograms corresponding to the sum of m/z 135.0301 ($[\text{Gua} - \text{NH}_3]^+$) and m/z 153.0411 ($[\text{Gua} - \text{NH}_3 + \text{H}_2\text{O}]^+$) were used for quantitation of EB-GII, while m/z 139.0183 ($[^{15}\text{N}_5\text{-Gua} - \text{NH}_3]^+$) and m/z 157.0283 ($[^{15}\text{N}_5\text{-Gua} - \text{NH}_3 + \text{H}_2\text{O}]^+$) at 5 ppm were used for quantitation of $[^{15}\text{N}_5]\text{-EB-GII}$. A full scan event was also performed over the mass range of m/z 100–500 at a resolution of 15,000 to monitor for any co-eluting matrix components. EB-GII amounts were determined by comparing the areas of the nanoLC/ESI⁺-HRMS³ peaks corresponding to the analyte and its internal standard using standard curves generated by analyzing known analyte amounts. The final EB-GII urine concentrations were normalized to urinary creatinine levels (determined separately, data not shown) to account for interindividual variability in urine flow rate and dilution.

Method validation

Standard curves were constructed by analyzing aqueous solutions containing fixed amounts of $^{15}\text{N}_5\text{-EB-GII}$ (5 fmol) and increasing amounts of EB-GII (0.1 – 10.0 fmol, in triplicate), followed by regression analyses. Method validation curves were constructed by spiking nonsmoker urine or artificial urine (200 μL) with 0.1 – 10 fmol of EB-GII and 5 fmol of $^{15}\text{N}_5\text{-EB-GII}$ internal standard (in triplicate), followed by sample processing and nanoLC/ESI⁺-HRMS³ analysis as described above. The observed EB-GII amounts were plotted against the theoretical values and the accuracy of the observed values was determined as described using the formula below.

The limit of detection (LOD) of the new method was determined by spiking synthetic urine (200 μL) with EB-GII (0.05, 0.1, 0.2, or 0.5 fmol) and $^{15}\text{N}_5\text{-EB-GII}$ (5 fmol). The fortified urine samples were processed and analyzed as described above. The method's limit of quantification (LOQ) was determined by spiking control nonsmoker urine (200 μL) with 0.1– 0.5 fmol of EB-GII and 5 fmol of $^{15}\text{N}_5\text{-EB-GII}$ internal standard (in triplicate), followed by sample processing and nanoLC/ESI⁺-HRMS³ analysis by standard methods. The LOD of the method was determined as the EB-GII amount that resulted in a signal to noise (S/N) ratio > 3, while the LOQ value was determined as the EB-GII amount which consistently gave a S/N ratio greater than 10 and a percent coefficient of variation (% CV) < 15%.

Intra- and inter-day precision and the accuracy of the analytical method were determined by spiking control nonsmoker urine (200 μL) with 0.2 fmol of EB-GII and 5.0 fmol of $^{15}\text{N}_5\text{-EB-GII}$ internal standard. The samples were analyzed three times per day on three consecutive days. The relative standard deviations between these measurements were calculated and reported as intra-day and inter-day precision, while the method accuracy range was determined 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 amounts added.

To determine analyte recovery from SPE, synthetic urine (200 μL , in triplicate) was spiked with $^{15}\text{N}_5\text{-EB-GII}$ internal standard (5 fmol). Unlabeled EB-GII standard (4.2 fmol) was added either prior to or immediately after SPE purification, followed by sample processing

by standard methods and nanoLC/ESI⁺-HRMS³ analysis as described above (in triplicate). SPE recovery was determined by comparing the theoretical and the observed ¹⁵N₅-EB-GII:EB-GII peak area ratios. Analyte signal suppression in urine matrix was evaluated from the ratio of peak area of EB-GII (4.2 fmol) spiked into artificial urine matrix post sample processing, and the corresponding peak area of the EB-GII obtained by nanoLC/ESI⁺-HRMS³ analysis of a pure standard in water.

Freeze-thaw analyte stability in human urine was determined using a smoker urine sample. The urine was frozen for 24 hours at -20 °C and thawed for 2 hours at room temperature (freeze-thaw cycle 1), after which it was processed and analyzed in duplicates. This procedure was repeated for two additional freeze-thaw cycles, followed by sample processing and analysis. Freeze-thaw cycle variability was determined using % CV within and between the cycles.

HPLC-ESI⁻-MS/MS analysis of urinary MHBMA and DHBMA

Urinary concentrations of MHBMA and DHBMA were determined using previously published methods.²⁶ Briefly, human urine (200 µL) was mixed with 200 µL of water, 20 µL of 1 N HCl, and 60 ng each of ²H₆-MHBMA and ²H₇-DHBMA (internal standards for mass spectrometry). Samples were subjected to solid phase extraction on a Waters Oasis HLB 96 well plate (1 mL/30 mg, Waters, Corp. Milford, MA). The cartridges were conditioned with 1 mL methanol, followed by 1 mL of water. Following sample loading, the cartridges were washed with 1 mL of 5% methanol. MHBMA, DHBMA, and their respective internal standards were eluted with 1.2 mL of 75% methanol, dried under vacuum, and reconstituted in 30 µL of 0.1% formic acid.

HPLC-ESI⁻-MS/MS analyses of MHBMA and DHBMA were performed on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a 96 well plate autosampler interfaced to a Thermo TSQ Vantage mass spectrometer (Thermo Scientific Corp., Waltham, MA). Samples were loaded onto an Agilent Pursuit 3 Diphenyl column (3 µm, 2.0 × 150 mm, Agilent Technologies, Santa Clara, CA) fitted with an Agilent Metaguard Pursuit 3 DP guard column (Agilent Technologies, Santa Clara, CA). The column was maintained at 5 °C and eluted with a gradient of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 150 µL/min. The gradient started at 3% B, was linearly increased to 9% B in 12 minutes, and further increased to 50% B in 2 min. The percentage of solvent B was maintained at 50% B for 2 min, increased to 80% B in 2 min, and held at 80% for another 2 min. Finally, the solvent composition was returned to 3% B in 2 min and equilibrated for 12 min. The following MS/MS transitions were used for selected reaction monitoring: MHBMA, *m/z* 232.11 → 103.11, ²H₆-MHBMA, *m/z* 238.15 → 109.15, DHBMA, *m/z* 250.10 → 75.13, and ²H₇-DHBMA, *m/z* 257.16 → 78.13. Additional details regarding mass spectrometer settings can be found in a prior publication.²⁶ MHBMA and DHBMA amounts were determined by comparing analyte peak areas with those of their respective internal standards using standard curves.

Genotyping

Smokers from the above ethnic differences study were genotyped as described below. The genetic variants were identified at the University of Hawaii Cancer Center Genomics Shared Resource. *CYP2E1* single nucleotide polymorphisms (SNPs) rs2070676 and rs6413432 were genotyped with a predesigned and custom TaqMan assays, respectively. Both of the *EPHX1* SNPs, rs1051740 and rs2234922, were genotyped with predesigned TaqMan assays, as was the rs9462088 (*FANCE*). The *GSTT1* copy number assay was run using TaqMan copy number assay Hs00010004_cn.

All assays were run on the 7900HT FAST Real-Time System (Life Technologies, Carlsbad, CA). SNPs were called using the TaqMan Genotyper software, and copy number calls were determined using the CopyCaller v2.0 software (Life Technologies, Carlsbad, CA). Approximately 5% blind duplicate samples were included for quality control.

Statistical Analysis

Least-square means (or geometric means) were estimated and compared for urinary EB-Guanine II, MHBMA and DHBMA levels between populations, smoking status or exposure levels. Several candidate SNPs in *CYP2E1*, *EPHX1* and *FANCE* genes, along with a *GSTT1* copy number genotype were also analyzed with geometric mean levels of urinary EB-Guanine II, MHBMA and DHBMA. All associations were evaluated using linear regression models, with adjustment for age, sex, (total nicotine equivalents, and race where appropriate), with a significance threshold at $p < 0.05$. To examine correlations between urinary EB-Guanine II levels and concentrations of BD-mercapturic acids, Pearson's partial correlations (r) were reported and adjusted for age, sex, total nicotine equivalents, and race.

Results

NanoLC/ESI⁺-HRMS³ method development for EB-GII in human urine

Human urine is a highly complex and variable biological matrix containing many polar metabolites, bile salts, and other small molecules that can adversely affect urinary DNA adduct analysis due to ESI signal suppression.^{27,28} Therefore, analyte-selective enrichment strategies such as SPE and/or offline HPLC fractionation are required for trace level analyses of urinary DNA adducts.^{29,30} Our experimental approach for quantitation of EB-GII in human urine is summarized in Scheme 2. Following spiking with ¹⁵N₅-EB-GII internal standard, urine samples were subjected to solid phase extraction on Strata X cartridges to eliminate the bulk of impurities, followed by further sample enrichment by offline HPLC prior to quantitative analysis by nanoLC/ESI⁺-HRMS³. The use of isotopically labeled internal standard early in the analysis accounted for any analyte losses during sample processing, allowing for accurate quantitation.

In the initial stages of method development, several types of SPE cartridges, including Strata X (Phenomenex), Oasis HLB (Waters Corp.), and Isolute ENV+ (Biotage), were tested for EB-GII isolation from urine matrix. Among these, Strata X was the most effective with respect to the elimination of co-eluting impurities and analyte recovery (SPE recovery, 56.6 ± 1.72 % in spiked urine samples).

Our initial experiments revealed that SPE alone did not afford sufficient sample purity for nanoLC-MS analysis of EB-GII. Therefore, SPE enriched fractions were subjected to additional enrichment by an offline HPLC method developed in an earlier study.¹⁹ HPLC fractions containing EB-GII and its internal standard were collected, concentrated under vacuum, and reconstituted with 10 μ L of water for nanoLC/ESI⁺-HRMS³ analysis. HPLC blanks were injected every 10–15 samples to detect any analyte carryover during the offline HPLC clean up procedure.

NanoLC/ESI⁺-HRMS³ analyses were performed using an Orbitrap Velos mass spectrometer. We took advantage of the accurate mass capabilities of the instrument in order to maximize the specificity and sensitivity of EB-GII detection in human samples.¹⁹ The use of the MS³ mode provided an increased specificity for the analyte and a reduced background noise, leading to excellent S/N ratios. NanoLC/ESI⁺-HRMS³ of standard solutions containing 0.1–10.0 fmol of EB-GII and 5.0 fmol of ¹⁵N₅-EB-GII each in 25 μ L of water produced linear standard curves ($y = 1.0044x$, $R^2 = 0.9984$) (Figure S1 in *Supporting Information*).

The new nanoLC/ESI⁺-HRMS³ method was further validated by analyzing EB-GII (0.1–10 fmol) and ¹⁵N₅-EB-GII (5 fmol) standards spiked into artificial urine or nonsmoker urine (200 μ L), followed by sample processing and analysis by standard methodologies. A linear correlation was observed between the actual and the calculated amounts of EB-GII in urine matrix ($y = 1.0042x$, $R^2 = 0.992$ for nonsmoker urine and $y = 0.9649x$, $R^2 = 0.994$ for artificial urine) (see Figures 1 and S2 in *Supporting Information*). Method LOD and LOQ values determined from the analysis of spiked samples were 0.25 fmol/mL and 0.5 fmol/mL urine, respectively (Figure S3A in *Supporting Information*). Intra-day and inter-day assay precision was determined by repeated analysis of EB-GII (0.2 fmol) spiked into nonsmoker urine (200 μ L) and was 13.1% and 10.6%, respectively (% CV, Table 1). Minimal signal suppression (< 25%) was observed upon comparison of nanoLC/ESI⁺-HRMS³ peak areas in the presence and in the absence of urine matrix. Method accuracy was determined to be $109.9 \pm 10.4\%$ when 0.2 fmol of EB-GII and 5 fmol of ¹⁵N₅-EB-GII were spiked into 200 μ L of nonsmoker urine (N = 5). Accuracy of varying concentrations of EB-GII (0.1–10 fmol) spiked into nonsmoker and artificial urine from the validation curves is between 83.9 – 115.4%. Freeze-thaw stability studies of a smoker urine sample (200 μ L, 2.45 ± 0.34 fmol) was < 15% RSD. Overall, our validation experiments have confirmed that the new analytical method was sensitive, accurate, and specific for EB-GII.

Quantitation of EB-GII in urine of F344 rats exposed to BD by inhalation

The validated nanoLC/ESI⁺-HRMS³ method was initially used to quantify EB-GII in urine of F344 rats treated with 0, 62.5, or 200 ppm BD by inhalation for 2 weeks (6 h/day, 5 days/week). Urinary EB-GII amounts increased in a concentration-dependent manner (Figure 2). Low but detectable amounts of EB-GII (14.5 – 87.0 pg/mL urine) were found in urine of control rats exposed to filtered air only (Figure 2). This finding is consistent with our earlier work which showed the presence of EB metabolites [2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(*N*-acetyl-L-cystein-S-yl)-2-hydroxy-but-3-ene, together known as MHBMA], in the urine of unexposed animals and non-smoker human subjects.²² Mean concentrations of EB-GII adducts in urine of male F344 rats exposed to 62.5 or 200 ppm BD

were 679.3 ± 86.3 and 1567.4 ± 401.5 pg/mL urine, respectively ($p = 0.06$, unpaired two sample t-test, Figure 2). Interestingly, urinary EB-GII concentrations were 1.5 times higher in female rats as compared to males, with 1311.7 ± 413.9 and 2672.4 ± 1005.69 pg EB-GII/mL urine present following exposure to 62.5 and 200 ppm BD, respectively (see Figure 2). While these results suggest possible gender differences in urinary BD-DNA adduct formation, differences in EB-GII excretion by male versus female rats were not statistically significant ($p = 0.12$, unpaired two sample t-test).

Quantification of EB-GII in urine of smokers, nonsmokers, and occupationally exposed workers

Our validated nanoLC/ESI⁺-HRMS³ methodology was next employed to determine urinary EB-GII concentrations in confirmed nonsmokers ($N = 28$), smokers ($N = 29$), administrative control workers ($N = 28$), and occupationally BD exposed workers ($N = 23$) from a BD production facility. NanoLC/ESI⁺-HRMS³ analyses have revealed clear EB-GII signals, with good S/N ratios and excellent chromatographic peak shapes, in most of the human urine samples analyzed (see examples in Figure 3A, B). In contrast, we were unable to quantify the EB-GI regioisomer due to a co-eluting peak, which interfered with analysis.¹⁹ This co-eluting impurity (m/z of 222.1681) had a different MS/MS fragmentation pattern than EB-G adducts of interest, but shared the same major fragment ion at m/z 152.0566, which interfered with accurate quantitation of EB-GI in human samples. Since our efforts to separate EB-GI from this contaminant by modifying HPLC conditions were unsuccessful, we focused on the EB-GII isomer. Previous studies have shown that EB-GI and EB-GII are formed in equal amounts in laboratory animals treated with BD,¹² and therefore either isomer can be used in molecular dosimetry studies.

Concentrations of EB-GII in urine of nonsmokers and smokers were 0.39 ± 0.13 and 0.59 ± 0.18 pg/mg creatinine, respectively (Table 2A). These differences were not statistically significant ($p = 0.399$), probably due to the large inter-individual variations and the small sample size ($N = 28$ and 29 , respectively, Table 2A). In contrast, urinary concentrations of EB-GII in urine of workers occupationally exposed to 0.1–2.2 ppm BD ($N = 23$) were significantly higher than in urine of administrative controls ($N = 28$) exposed to <0.01 ppm BD (0.22 ± 0.08 vs 1.25 ± 0.51 pg/mg creatinine, respectively; see Table 2B) ($p = 0.0024$).

Ethnic Differences in EB-GII excretion

African American smokers are at a higher risk for the development of lung cancer as compared to European American and Japanese American individuals.³¹ It has been proposed that these ethnic differences in sensitivity could be due to differences in carcinogen exposure and/or metabolism. African American smokers have been reported to excrete higher amounts of mercapturic acids derived from acrolein, crotonaldehyde, and benzene.^{32,33} To reveal any potential ethnic differences in BD metabolism and DNA adduct formation, urinary EB-Gua concentrations and BD-mercapturic acids were quantified in smokers belonging to two different ethnic groups: African American and European American. After assessing data completion for all adjustment variables, urinary EB-GII concentrations were determined in male ($N = 76$) and female ($N = 73$) smokers belonging to African American ($N = 74$) and European American ($N = 75$) (Table 3A). European American smokers

excreted significantly higher concentrations of EB-GII (0.48 ± 0.09 pg/mg creatinine) as compared to African American smokers (0.12 ± 0.02 pg/mg creatinine), respectively, adjusted for age, sex, and total nicotine equivalents (TNE). This difference was significant (p value = 3.1×10^{-7}). In contrast, no statistically significant differences were observed between male and female smokers. Furthermore, no correlations were observed between EB-GII levels and smoker's age, CO concentration, and cigarettes per day.

To determine whether the observed ethnic differences in urinary BD-DNA adducts can be attributed to differences in BD metabolism, we quantified urinary concentrations of BD metabolites MHBMA and *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine (DHBMA) in the same individuals (Table 3B). We found that European American smokers excreted significantly higher amounts of MHBMA than African Americans (15.70 ± 1.71 vs. 7.39 ± 0.82 ng/mg creatinine, respectively, adjusted for age, sex, and TNE; $p = 3.6 \times 10^{-6}$) (Table 3B). In contrast, urinary concentrations of DHBMA did not significantly differ between the two groups; European Americans excreted 283.71 ± 18.07 ng/mg creatinine, while African Americans excreted 236.98 ± 15.30 ng/mg creatinine ($p = 0.051$, adjusted for age, sex, and TNE, Table 3C). Although the same trend was observed for BD metabolites and urinary DNA adducts in these two ethnic groups, statistical analyses have revealed a weak association between urinary EBG adduct levels and the concentrations of BD-mercapturic acids (EB-GII with MHBMA, $R = 0.035$; EB-GII with DHBMA, $R = 0.028$).

To determine whether the inter-individual differences in EB-GII concentrations were caused by genetic polymorphisms in carcinogen metabolism and DNA repair enzymes, smokers from the two ethnic differences study were genotyped for GSTT1 copy number and common SNPs in *CYP2E1* monooxygenase (rs2070676, rs6413432), microsomal epoxide hydrolase, *EPHX1* (rs2234922, rs1051740), and *FANCE* DNA repair gene (rs9462088). After adjusting for age, gender, race, and TNE, we identified a significant association between urinary MHBMA levels and GSTT1 copy number ($p = 6.97 \times 10^{-10}$, Table 4). In contrast, no significant correlations were found between urinary excretion of EB-GII and polymorphisms in *CYP2E1*, *EPHX1*, or *FANCE* genes, most likely due to the relatively small sample size (Table 4).

Discussion

Covalent BD-nucleobase adducts within genomic DNA can be utilized as biologically relevant biomarkers of BD exposure and metabolic activation to reactive epoxides.^{34–38} These adducts represent the biologically relevant carcinogen dose following exposure to BD, and appear to correlate with cancer development in experimental animal models.^{12,35} We recently reported mass-spectrometry based quantitation of N-7-(1-hydroxy-3-buten-2-yl) guanine (EB-GII) adducts induced by EB (a major genotoxic metabolite of BD) in DNA isolated from peripheral blood leukocytes of smokers, nonsmokers and occupationally exposed BD workers.¹⁹ Although EB-GII was detectable in blood DNA of most human subjects, its concentrations were below the LOQ of the method (0.4 adducts/ 10^9 nucleotides or 0.2 fmol/150 μ g of DNA). N7-guanine adducts such as those induced by BD-derived epoxides are hydrolytically unstable due to the presence of a positive charge on the N7 position of the alkylated base, and are spontaneously released from the DNA backbone over

time (Scheme 1).^{12,36,37} We therefore hypothesized that EB-GII adducts could be detected in urine of exposed individuals and used as a biomarker of BD exposure.

Urinary DNA adducts represent useful biomarkers of carcinogen exposure and metabolic activation to electrophilic species. DNA adducts can be released from the DNA backbone and excreted in urine as free nucleobases as a result of their spontaneous depurination^{15,39} or their active repair via base excision repair pathways.⁴⁰ Previous studies have detected urinary nucleobase adducts induced by estrogens,⁴¹ lipid peroxidation products,⁴² *N*-ethyl-*N*-nitrosourea,³⁹ and reactive metabolites of environmental chemicals such as polyaromatic hydrocarbons⁴³ and styrene.⁴⁴ Although nucleobase adducts found in urine may have sources other than DNA (for example, from RNA or cellular nucleoside pools), urinary adducts can serve as a biomarker of carcinogen exposure and metabolic activation to DNA-reactive species.

In the present work, we have developed a quantitative method for EB-GII adducts in human urine, which is readily available and is easier to collect and store than blood or tissue samples. Since urine is a highly complex and variable biological matrix as compared to DNA, advanced sample enrichment procedures were used to isolate urinary adducts of interest, followed by sensitive and specific analysis by nanoLC/ESI⁺-HRMS.³

EB-GII adducts were first quantified in urine of male and female F344 rats exposed to BD by inhalation. We found that urinary EB-GII adduct numbers correlated in a dose-related fashion with BD exposure concentrations, with increasing numbers of adducts observed in animals exposed to higher concentrations of BD (Figure 2). Interestingly, detectable levels of EB-GII (14.5 – 87.0 pg/mL urine) were observed in urine of control rats exposed to filtered air (Figure 2), suggesting that there might be an endogenous source for this adduct.⁴⁵ This finding is in accord with our observation of the BD-mercapturic acids MHBMA and DHBMA in urine of control F344 rats.²⁶ Furthermore, significant quantities of the corresponding EB-specific hemoglobin adducts, *N*-(2-hydroxy-3-butenyl)-valine (HB-Val), were previously found in unexposed F344 rats.⁴⁶ It has been hypothesized that MHBMA and other four-carbon chain metabolites can originate from lipid peroxidation pathways or catabolism of carbohydrates, which could account for endogenous EB formation.^{47–49} Regardless, urinary EB-GII levels were strongly associated with BD exposure concentrations (Figure 2), validating their use as biomarkers of exposure to BD.

EB-GII adducts were readily detectable in human urine (Figure 3), with adduct concentrations ranging between 0.03 and 14 pg/mg creatinine. The use of accurate mass MS³ methodology on an Orbitrap mass spectrometer allowed us to obtain MS³ spectra of urinary EB-GII from human samples, which matched that of an authentic standard (Figure 3). Accurate quantitation was achieved by using isotope dilution with the corresponding ¹⁵N-labeled standard. The validated nanoLC/ESI⁺-HRMS³ method has an accuracy of 109.9 ± 10.4% and intra- and inter-day precision (% CV) less than 13.1% for 0.2 fmol spiked in 200 µL of nonsmoker urine. A comparison of EB-GII levels in the urine of occupationally exposed workers and control administrative workers (Table 2B) provides evidence for increased adduct numbers in occupationally exposed individuals (*p* = 0.0024), consistent with differences in BD exposure. This finding indicates that urinary EB-GII

adduct levels can be used as mechanism-based biomarkers of BD exposure in humans. EB-GII adduct levels were also higher in confirmed smokers (0.59 pg/mg creatinine) as compared to nonsmokers (0.39 pg/mg creatinine), although these differences were not statistically significant (Table 2A).

Determination of the true amounts of BD exposure in smokers is difficult due to variability in factors such as puff volume and duration, inter-puff interval, and number of puffs per cigarette, all of which are based on the extent of nicotine addiction.^{50,51} Soeteman-Hernández et al. estimated that smokers consuming an average of 20 cigarettes/day are exposed to 0.01 mg BD/kg/day (assuming a mean concentration of 128 µg BD per cigarette) and that the amount of the dose that reaches the alveoli is 70%, with the extent of alveolar absorption being 50%.⁵² For example, a 70 kg individual would consume a total of 0.7 mg BD/day.⁵² In the case of occupationally exposed workers, OSHA limits BD exposure to 1.0 ppm (2.21 mg/m³) per 8-hour workday.² Assuming an average human inhalation volume of 20 m³/day, occupational BD exposure would be approximately 44.2 mg/day, which is 60-fold higher than the amount inhaled by smokers.⁵² Average BD urinary metabolite concentrations of MHBMA, DHBMA, and 4-(*N*-acetyl-L-cystein-S-yl)-1,2,3-trihydroxybutane (THBMA) in occupationally exposed workers were 82 ± 138, 3094 ± 4162 and 157 ± 158 ng/mg creatinine, respectively.²² In smokers, the corresponding concentrations were 11 ± 12, 631 ± 452, and 31 ± 20 ng/mg creatinine, respectively,²² reflecting lower BD exposures in the latter.

Smokers belonging to various ethnic groups have been reported to have a different risk of lung cancer development, with the relative risk being highest among African American and Native Hawaiian smokers, followed by European American, Japanese American, and Latino smokers.³¹ It is possible that these differences are at least in part attributed to ethnic differences in carcinogen metabolism and DNA adduct formation and repair. This prompted us to quantify both urinary BD metabolites and DNA adducts associated with BD exposure to gain insight into the underlying cancer risk in smokers from two different ethnic groups: African Americans and European Americans. Overall, urinary EB-GII levels were significantly higher in European American smokers as compared to African American smokers ($p = 3.1 \times 10^{-7}$, adjusted for age, sex, and TNE, Table 3A). Furthermore, urinary MHBMA levels were ~2-fold higher in European American smokers as compared to African American smokers ($p = 3.6 \times 10^{-6}$, adjusted for age, sex, and TNE, Table 3B), while urinary DHBMA levels, although slightly higher in European American smokers, did not significantly differ between the two groups ($p = 0.051$, adjusted for age, sex, and TNE, Table 3C). These results are similar to those previously reported by our laboratory, in which European American smokers were found to have significantly higher urinary concentrations of MHBMA as compared to Native Hawaiian and Japanese American smokers, whereas urinary DHBMA concentrations did not significantly differ between these groups.⁵³

Comparing urinary EB-GII levels to urinary MHBMA and DHBMA levels has revealed that there was no significant correlation between EB-GII and BD-mercapturic acids ($R \sim 0.03$). Since urinary EB-GII levels represent the concentration of BD DNA adducts released from DNA due to spontaneous hydrolysis and/or DNA adduct repair, increased adduct amounts in European American smokers could represent more efficient removal of EB-GII adducts by

European American as compared to African American smokers. Further studies involving larger numbers of European American and African American smokers, as well as subjects from additional ethnic groups such as Latinos, Japanese Americans, and Native Hawaiians, should be conducted to confirm this result.

Genotyping analysis (Table 4) indicated that urinary MHBMA is strongly associated with GSTT1 copy number. GSTT1 is a major enzyme in BD metabolism and is responsible for detoxification of BD epoxides, including EB (Scheme 1).⁵³ In contrast, no associations were observed between urinary EB-GII, MHBMA, or DHBMA and frequently occurring SNPs on BD metabolizing genes *CYP2E1* (rs2070676, rs6413432) and *EPHX1* (rs2234922, rs1051740), or the DNA repair gene *FANCE* (rs9462088) (Table 4). These observations are likely due to the small size of our study population (N = 151) and the limited number of variants considered in the analysis (Table 4).

In summary, we have developed and validated an isotope dilution nanoLC/ESI⁺-HRMS³ method for urinary EB-GII adducts of BD in humans. This method has excellent sensitivity, accuracy, and selectivity. Our results indicate that smokers and occupationally exposed individuals have a greater exposure to known human carcinogen BD than nonsmoking individuals. Furthermore, significant ethnic differences in urinary levels for EB-GII in smokers were uncovered, which could be partially explained by GSTT1 copy number. The methodologies developed in this work will be useful for future studies of BD exposure and metabolic activation in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Supporting information

Supplementary information is provided including accuracy and precision results for NanoLC/ESI⁺ HRMS³ analysis of EB-GII, NanoLC/ESI⁺-HRMS³ method validations (correlations between spiked and observed amounts of EB-GII spiked into water and artificial urine), and NanoLC/ESI⁺ HRMS³ analysis of EB-GII and [¹⁵N₅]-EB-GII spiked in nonsmoker urine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Abbreviations

BD	1,3-Butadiene
dT	2'-deoxythymidine
EB	3,4-epoxy-1-butene
THBMA	4-(<i>N</i> -acetyl-L-cystein-S-yl)-1,2,3-trihydroxybutane
CID	collision induced dissociation

HCD	high collision dissociation
nanoLC/ESI⁺-HRMS³	isotope dilution nanoLC-high resolution tandem mass spectrometry
HB-Val	N-(2-hydroxy-3-butenyl)-valine
EB-GII	N7-(1-hydroxy-3-buten-2-yl) guanine
EB-GI	N7-(2-hydroxy-3-buten-1-yl) guanine
DHBMA	<i>N</i> -acetyl- <i>S</i> -(3,4-dihydroxybutyl)-L-cysteine
HMVK	Hydroxymethylvinyl ketone
GSTT1	Glutathione S-transferase (GST) theta 1
EH	Epoxide Hydrolase
CR	Carbonyl reductase
ADH	Alcohol dehydrogenase
MA	Mercapturic acid
SNPs	single nucleotide polymorphisms
SPE	solid phase extraction
TNE	total nicotine equivalents

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Figure 1.

NanoLC/ESI⁺-HRMS³ method validation: correlation between the spiked and the observed amounts of EB-GII spiked into 200 μ L of nonsmoker urine. Spiked amounts were 0, 0.1, 0.2, 0.5, 1, 5, or 10 fmol of EB-GII and 5 fmol of ¹⁵N₅- EB-GII (internal standard), followed by sample processing and MS analysis. Insert shows correlation between spiked and observed amounts of EB-GII at the lower range (0.1–1.0 fmol).

Figure 2. Concentration-dependent formation of EB-GII adducts in F344 male and female rats treated with increasing amounts of EB (0, 62.5, 200 ppm).

Figure 3.

Representative extracted ion chromatogram, EB-GII and ¹⁵N₅-EB-GII MS³ ion fragmentation spectra for nanoLC/ESI⁺-HRMS³ analysis of EB-GII adducts in smoker (**A**, **C**), occupationally exposed BD worker urine (**B**, **D**) (200 μ L urine each) spiked with ¹⁵N₅-EB-GII (5 fmol internal standard for quantitation) and subjected to sample processing, and nanoLC/ESI⁺-HRMS³ analysis on an Orbitrap Velos mass spectrometer.

Scheme 1.

Partial metabolic scheme of BD showing DNA adduct formation, hydrolysis and urinary excretion. 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.

Sample preparation procedure for isotope dilution nanoLC/ESI⁺ HRMS³ analysis of EB-GII in urine.

Table 1

Accuracy and precision results for NanoLC/ESI⁺ HRMS³ analysis of EB-GII (0.2 fmol) spiked into 200 μ L of nonsmoker urine.

Day 1	Mean	0.19
	RSD (%)	13.1
	Accuracy(%)	96.4
	N	3
Day 2	Mean	0.19
	RSD (%)	11.7
	Accuracy(%)	97.5
	N	3
Day 3	Mean	0.21
	RSD (%)	9.9
	Accuracy(%)	103.5
	N	3
Interday	Mean	0.19
	RSD (%)	10.6
	Accuracy(%)	99.2
	N	9

Table 2

The geometric mean urinary EB-GII (pg/mg creatinine) values for nonsmokers, smokers, administrative control workers, and occupationally exposed workers.

EB Guanine II (pg/mg creatinine)			
A	N	Geometric Mean $\pm$ SD [*]	p-value [*]
Nonsmokers	28	0.39 $\pm$ 0.13	0.399
Smokers	29	0.59 $\pm$ 0.18	
EB Guanine II (pg/mg creatinine)			
B	N	Geometric Mean $\pm$ SD	p-value
Control Workers	28	0.22 $\pm$ 0.08	0.0024
Exposed Workers	23	1.25 $\pm$ 0.51	

* The geometric means on the log transformed variable and the p-values are adjusted for age and sex

Table 3

The geometric means for urinary EB-GII, MHBMA, and DHBMA levels (pg/mg creatinine) in African American and European American smokers.

EB Guanine II (pg/mg creatinine)			
A	N	Geometric Mean ± SD *	Adjusted p-value*
African Americans	74	0.12 ± 0.02	3.1 × 10 ⁻⁷
European American	75	0.48 ± 0.09	
MHBMA (ng/mg creatinine)			
B	N	Geometric Mean ± SD *	Adjusted p-value *
African Americans	75	7.39 ± 0.82	3.6 × 10 ⁻⁶
European American	76	15.70 ± 1.71	
DHBMA (ng/mg creatinine)			
C	N	Geometric Mean ± SD *	Adjusted p-value *
African Americans	74	236.98 ± 15.30	0.051
European American	75	283.71 ± 18.07	

* The geometric means on the log transformed variables and the p-values are adjusted for age, sex and total nicotine equivalents.

Table 4

Geometric mean values of urinary EB-GII, MHBMA, and DHBMA stratified by GSTT1 copy number genotype and candidate SNPs in *CYP2E1*, *EPHX1*, and *FANCE*.

N = 151	MHBMA (ng/mg creatinine)			DHBMA (ng/mg creatinine)			EB-Guanine II (pg/mg creatinine)		
	N	Geometric Mean [Std Err]*	p-value*	N	Geometric Mean [Std Err]*	p-value*	N	Geometric Mean [Std Err]*	p-value*
rs1051740									
0	99	11.09 [1.06]		99	248.86 [14.03]		97	0.23 [0.04]	
1	41	11.41 [1.72]	0.564	41	278.52 [24.69]	0.4961	41	0.29 [0.07]	0.613
2	8	7.52 [2.74]		8	287.63 [61.70]		8	0.16 [0.10]	
rs2234922									
0	84	11.72 [1.24]		84	263.45 [16.39]		83	0.22 [0.04]	
1	55	9.99 [1.30]	0.631	55	247.88 [18.90]	0.5433	54	0.23 [0.05]	0.1882
2	11	10.15 [2.95]		11	301.23 [51.54]		11	0.56 [0.26]	
rs2070676									
0	62	9.45 [1.41]		62	252.82 [22.81]		61	0.37 [0.09]	
1	50	11.10 [1.49]	0.443	50	284.11 [23.11]	0.3784	50	0.17 [0.04]	0.085
2	37	13.37 [2.44]		37	242.72 [26.78]		36	0.19 [0.06]	
rs9462088									
0	103	11.14 [1.07]		103	272.82 [15.46]		102	0.27 [0.04]	
1	37	10.90 [1.76]	0.379	37	242.56 [23.24]	0.2736	37	0.17 [0.04]	0.277
2	10	7.06 [2.16]		10	206.07 [37.36]		9	0.31 [0.16]	
rs6413432									
0	128	10.58 [0.90]		128	258.00 [12.81]		126	0.27 [0.04]	
1	19	11.73 [2.58]	0.663	19	285.76 [36.67]	0.4769	19	0.20 [0.07]	0.412
2	—	—		—	—		—	—	
GSTT1_CN									
0	36	4.95 [0.68]		36	232.44 [22.12]		35	0.29 [0.07]	
1	54	9.98 [1.12]	6.97 × 10⁻¹⁰	54	270.58 [20.94]	0.5713	53	0.22 [0.05]	0.552

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N = 151		MHBMA (ng/mg creatinine)		DHBMA (ng/mg creatinine)		EB-Guanine II (pg/mg creatinine)				
		N	Geometric Mean [Std Err]*	p-value*	N	Geometric Mean [Std Err]*	p-value*	N	Geometric Mean [Std Err]*	p-value*
2	51	51	18.55 [2.14]		51	270.82 [21.62]		51	0.25 [0.05]	
3	5	5	15.46 [5.71]		5	284.15 [72.53]		5	0.57 [0.39]	

*The geometric means and p-values are adjusted for age, gender, race, and total nicotine equivalents