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Radiation Metabolomics: Identification of Minimally Invasive Urine Biomarkers for Gamma-Radiation Exposure in Mice

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Abstract

Gamma-radiation exposure has both short- and long-term adverse health effects. The threat of modern terrorism places human populations at risk for radiological exposures, yet current medical countermeasures to radiation exposure are limited. Here we describe metabolomics for γ -radiation biodosimetry in a mouse model. Mice were γ -irradiated at doses of 0, 3 and 8 Gy (2.57 Gy/min), and urine samples collected over the first 24 h after exposure were analyzed by ultra-performance liquid chromatography–time-of-flight mass spectrometry (UPLC–TOFMS). Multivariate data were analyzed by orthogonal partial least squares (OPLS). Both 3- and 8-Gy exposures yielded distinct urine metabolomic phenotypes. The top 22 ions for 3 and 8 Gy were analyzed further, including tandem mass spectrometric comparison with authentic standards, revealing that *N*-hexanoylglycine and β -thymidine are urinary biomarkers of exposure to 3 and 8 Gy, 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate is elevated in urine of mice exposed to 3 but not 8 Gy, and taurine is elevated after 8 but not 3 Gy. Gene Expression Dynamics Inspector (GEDI) self-organizing maps showed clear dose–response relationships for subsets of the urine metabolome. This approach is useful for identifying mice exposed to γ radiation and for developing metabolomic strategies for noninvasive radiation biodosimetry in humans.

INTRODUCTION

Humans are exposed to ionizing radiation from several sources. Natural background radiation from galactic and solar cosmic rays and terrestrial radionuclides of radon, potassium, uranium and thorium represents about 80% of the average radiation exposure to Americans of 3.6 mSv per year (1). The remaining exposure can be attributed to man-made radiation sources, including diagnostic X rays, nuclear medicine and radiotherapy. Finally, various consumer products are a source of radiation exposures; these include televisions, watches, carbon-based fuels, smoke detectors and fluorescent lamp starters. However, with the growing need for nuclear waste disposal into the environment and the ever-increasing threat of a terrorist nuclear event, it is now necessary to develop biomarkers of ionizing radiation exposure that can be used for mass screening in the event of a radiological mass casualty incident. The identification of dosimetry biomarkers is a priority effort in preparation for a possible terrorist attack with radiological or nuclear devices (2,3). High-throughput appliances that could evaluate such

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exposures are envisioned as being employed to guide triage and subsequent therapeutic choices (2–4).

The search for biomarkers of effective dose and the early effects of ionizing radiation exposure in both humans and experimental animals has a history spanning several decades. Blood cells and serum have proven to be abundant sources of human radiation biomarkers, including those of DNA damage and repair (5,6), chromosomal aberrations (7), DNA-protein crosslinks (8), red blood cell polyamine levels (9), serum proteomic profiles (10), and gene expression profiles determined by both microarrays (11) and RT-PCR (12).

Urine analysis has also provided insights into metabolic perturbations associated with radiation exposure and it has the added advantage of giving a metabolic picture over time because metabolites accumulate in the bladder and can be collected and pooled over set periods, as opposed to the snapshot obtained from a single blood sample. Historically, efforts have concentrated largely on neurotransmitters and their metabolites on the premise that stress, including radiation stress, should trigger the release of neurotransmitter molecules. Examples include 5-hydroxyindoleacetic acid (13–17), indoxyl sulfate (15), 3-methoxy-4-hydroxymandelic acid, 3-methoxy-4-hydroxyphenylglycol, metanephrine, normetanephrine, and homovanillic acid (18). More recently, urinary markers of DNA damage and repair have been identified, including thymine glycol (19,20), thymidine glycol (19), and 8-hydroxyguanine (20). Other potential urinary biomarkers of ionizing radiation that have been discussed include thromboxane (21) and 8-iso-prostaglandin $F_{2\alpha}$ (22), although this latter example is controversial (23).

Many of the studies mentioned above were carried out in laboratory rodents. The approaches to identifying biomarkers for ionizing radiation damage have all been predicated on the basis of known or suspected biological effects of radiation such as neurotransmitter release, DNA damage or inflammation. Despite the considerable number of biological molecules involved in just these processes, the number of studies of different biomarkers of ionizing radiation has been modest. One study, however, investigated possible radiation injury with no prior hypothesis except that signs of disturbances in cellular metabolism caused by radiation exposure after the Chernobyl reactor incident should be detectable in urine. ^1H and ^{31}P NMR analysis of human urine revealed some poorly defined changes in “*N*-trimethyl groups” and in creatinine, citric acid, glycine and hippuric acid (24).

An examination of the pathways of cellular metabolism reveals that the low-molecular-weight (<600 Da) intermediates and end products of metabolism that are excreted in the urine are mainly acids, phenols, phenolic acids and amino acids. Purely basic compounds generally are not excreted in urine without metabolism to acidic or Zwitterionic metabolites. Therefore, urine contains a host of anionic substances, with humans excreting approximately 60 mmol organic acids per day with a mean urinary pH of approximately 6.0 (25). Metabolomics is a means of measuring small-molecule metabolite profiles and fluxes in biological matrices after genetic modification or exogenous challenges, and it has become an important component of systems biology, complementing genomics, transcriptomics and proteomics (26–28). The ability to register the increases and decreases in intermediary metabolites has progressed considerably due to advances in analytical chemical platforms for metabolite detection and quantification and in chemometric software for performing multivariate data analysis on very large data sets. As such, metabolomics can provide an unbiased evaluation of upward and downward metabolite fluxes. Negative-ion mass spectrometry is well suited to record the fluxes of urinary organic anions.

In this study, we have harnessed the high resolution capability of ultra-performance liquid chromatography (UPLC) coupled with the accurate mass determination of time-of-flight mass

spectrometry (TOFMS) and various multivariate data analyses to uncover metabolomic responses in mice γ -irradiated with either 3 or 8 Gy. Such radiation metabolomic signatures may be useful in designing protocols and novel methodologies for screening at-risk human populations and measuring radiation dose.

MATERIALS AND METHODS

Compounds

The following compounds were obtained from Sigma-Aldrich Co., St. Louis, MO: citric acid, isocitric acid, taurine, theophylline, 4-nitrobenzoic acid, creatinine, α -thymidine, β -thymidine, 2-, 3- and 4-hydroxyphenylacetic acid, mandelic acid (α -hydroxyphenylacetic acid), 3-, 4- and 5-methylsalicylic acid, 3-hydroxy-4-methylbenzoic acid, 4-hydroxy-2-methylbenzoic acid, and 4-hydroxy-3-methylbenzoic acid. 6-Methylsalicylic acid (2,6-cresotic acid), 3-hydroxy-2-methylbenzoic acid (3,2-cresotic acid), 3-hydroxy-5-methylbenzoic acid (3,5-cresotic acid), and 5-hydroxy-2-methylbenzoic acid (5,2-cresotic acid) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Stachadrine hydrochloride (*N*-methylproline, proline betaine) was obtained from Extrasynthese (Lyon, France), and *N*-hexanoylglycine was purchased from the Metabolic Laboratory, VU Medical Center (Amsterdam, Netherlands). All inorganic reagents and solvents were of the highest purity obtainable.

Generation of O-Sulfate Conjugates In Situ

2-, 3- and 4-Hydroxyphenylacetic acid, mandelic acid, 3-, 4- and 5-methylsalicylic acid, 3-hydroxy-4-methylbenzoic acid, 4-hydroxy-2-methylbenzoic acid, 4-hydroxy-3-methylbenzoic acid, 6-methylsalicylic acid (2,6-cresotic acid), 3-hydroxy-2-methylbenzoic acid (3,2-cresotic acid), 3-hydroxy-5-methylbenzoic acid (3,5-cresotic acid), and 5-hydroxy-2-methylbenzoic acid (5,2-cresotic acid) were all treated individually with concentrated H_2SO_4 (specific gravity 1.86) to generate their respective *O*-sulfate conjugates *in situ*. The experiments were designed to minimize the extent of aromatic ring sulfonation (29) by sulfating each acid (2–5 mg) in concentrated H_2SO_4 containing 10% water, both on ice and at room temperature. Aliquots were taken at 30, 60, 90 and 120 min and neutralized on ice by the careful addition of ammonium hydroxide solution. Resulting neutral solutions were analyzed by UPLC-TOFMS (see below).

Animals

Male C57BL6 mice, 11–20 weeks of age obtained from Charles River Laboratories, Inc. (Wilmington, MA) by way of NCI-Frederick (Frederick, MD), were used for this study. This strain is intermediate in radiation sensitivity, with 3–4-month-old male mice having a reported 30-day mean 5.7 and 7.3 Gy in the most radiosensitive LD_{50} of 6.5 Gy, compared to $<$ and radioresistant strains studied, BALB/cJ and 129/J, respectively (30). Mice received water were fed NIH31 chow *ad libitum* and were housed under a standard 12 h light/12 h dark cycle. All animal handling and experimental protocols were designed for maximum possible well-being, conformed to the guidelines stipulated by the National Institutes of Health Office of Animal Care and Use, and were approved by the NCI Animal Care and Use Committee prior to the initiation of this study.

Radiation Exposures

Groups of mice were exposed to doses of 3 ($n = 12$), 6 ($n = 8$), 7 ($n = 10$), 8 ($n = 10$ and 12), or 11 Gy ($n = 10$) γ radiation from a ^{137}Cs source in a Mark I Model 68 small animal irradiator (J. L. Shepherd & Associates, San Fernando, CA) operating at 2.57 Gy/min. The Mark I has

a single ^{137}Cs source and provides uniform doses to small animals centered on the revolving turntable revolving at a constant rate of 4.75 revolutions per minute within the chamber. We irradiated mice in a pie cage (25.5 cm inner diameter) that separates and distributes them evenly for uniform exposures. The source-to-surface distance in this configuration varies over time at a constant rate, averaging 20 cm with a range of 7.3 to 32.8 cm. The dose for a given mouse is uniform and is virtually identical to the dose of each cage mate. Physical doses within the chamber were assessed using model AT-742 (0–2 Gy) and AT-746 (0–6 Gy) direct reading dosimeters (Arrow-Tech, Inc., Rolla, ND).

A dose of 7 Gy in mice is generally considered to be equivalent to 4 Gy for humans (1). Thus the doses we used were roughly equivalent to doses of 1.7 and 4.6 Gy to humans. That is, the doses were either below or within the range (2.5–5 Gy) considered to be associated with the radiation hematopoietic syndrome (1). For identification of urine biomarkers, we chose doses of 3 and 8 Gy. The $\text{LD}_{50/30}$ for C57BL/6 mice in our laboratories is 7 to 8 Gy, so we chose 8 Gy to maximize any potential metabolomic response and 3 Gy as a sublethal dose generally associated with some cellular changes but no outward, noticeable symptoms or behavior changes (31).

Urine Collection

Urine samples from mice housed individually in Nalgene metabolic cages (Tecniplast USA, Inc., Exton, PA) were collected over continuous 24-h periods with alternate 24-h rest intervals. Urine was collected over 24 h to avoid the effects of diurnal variation on urine metabolite profiles shown by others (32,33). Three 24-h urine samples per mouse were obtained at 6, 4 and 2 days before exposure. Control mice were handled identically, including a sham irradiation with 0 Gy in the irradiator, and were used for simultaneous control urine sample collection. The sample collection protocol is illustrated in Fig. 1. All urine samples were stored at -80°C until analyzed.

Ultra-performance Liquid Chromatograph–Time-of-Flight Mass Spectrometry (UPLC-TOFMS) Analyses

The urine samples were analyzed by UPLC-TOFMS in order by time and by mouse number in the same continuous session to eliminate instrument bias as a potential confounder. The operator was blinded to the exposure status of the samples at the time of the UPLC-TOFMS analysis so that confounding by operator bias was minimized. Urine aliquots (50 μl) were diluted 1:5 with 50% aqueous acetonitrile (200 μl) and centrifuged at $13,000g$ for 20 min at 4°C to remove particulates and precipitated proteins. Aliquots (100 μl) of supernatant were transferred to auto-sampler vials for UPLC-TOFMS analysis and injected (5 μl) onto a reverse-phase 50×2.1 mm ACQUITY[®] 1.7- μm C18 column (Waters Corp, Milford, MA) using an ACQUITY[®] UPLC system (Waters) with a gradient mobile phase comprising 0.1% formic acid solution (A) and acetonitrile containing 0.1% formic acid solution (B). Each sample was resolved for 10 min at a flow rate of 0.5 ml/min. The gradient consisted of 100% A for 0.5 min, 20% B for 3.5 min, 95% B for 4 min, 100% B for 1 min, and finally 100% A for 1 min. The column eluent was introduced directly into the mass spectrometer by electrospray. Mass spectrometry was performed on a Q-TOF Premier[®] (Waters) operating in either negative-ion (ESI[−]) or positive-ion (ESI⁺) electrospray ionization mode with a capillary voltage of 3000 V and a sampling cone voltage of 30 V. The desolvation gas flow was set to 650 liters/h and the temperature was set to 350°C . The cone gas flow was 50 liters/h, and the source temperature was 120°C . Accurate mass was maintained by introduction of Lock-Spray[®] interface of sulfadimethoxine (309.0658 $[\text{M-H}]^{-}$) at a concentration of 250 pg/ μl in 50% aqueous acetonitrile and a rate of 30 $\mu\text{l}/\text{min}$. Data were acquired in centroid mode from 50 to 800 m/z in MS scanning. Tandem MS collision energy was scanned from 5 to 35 V.

Data Processing and Multivariate Data Analysis

Centroided and integrated mass spectrometry data from the UPLC-TOFMS were processed to generate a multivariate data matrix using MarkerLynx[®] (Waters). All data for each urine ion were normalized by relative creatinine concentrations on a per sample basis. Centroided data were Pareto-scaled and analyzed further by principal components analysis (PCA) and orthogonal partial least squares (OPLS) using SIMCA-P+ software (Umetrics, Kinnelon, NJ). Samples were classified as either from control ($y = 0$) or irradiated ($y = 1$) mice for OPLS used to determine which metabolites contribute most to the separation in the scores space and are thus elevated in urine samples from irradiated mice compared with samples from control mice. Selection of candidate markers was accomplished by examining the scatter S-plots of significance (P) as a function of weight, i.e., how much a particular ion correlated to the model and a measure of its relative abundance.

Identification of Metabolites

In each loading S-plot, the ions positioned most distant from the origin in the upper right quadrant were investigated further as candidate biomarkers of γ -radiation exposure. Twenty-two ions were then chosen based on S-plot coordinates and lowest P values derived from both two-tailed t tests (parametric) of the normalized means and Wilcoxon-Mann-Whitney tests (nonparametric) of the normalized data. Twenty-two ions were chosen in each experiment to characterize the similarities and differences in the responses to these two doses. Elemental compositions were generated with MarkerLynx based on the exact masses of the high-contribution score metabolites. Identification of the top metabolites was informed by biological relevance and likelihood of presence in the urine. Authentic standards at 20–60 μM in 50% acetonitrile and 0.1% formic acid were then used to confirm the identities of the markers with UPLC-MS/MS. Tandem MS (MS/MS) fragments the molecules in a consistent manner. Therefore, putative urine metabolites provide an MS/MS fragmentation spectrum identical to that of the known standards.

Quantification and Statistical Analysis of Urine Biomarkers

QuanLynx software (Waters) was used to quantify urine metabolites based on their peak areas. Calibration curves were constructed for authentic creatinine (MH^+ 114.0667 m/z), thymidine ($[\text{M}-\text{H}]^-$ 241.0824 m/z), *N*-hexanoylglycine ($[\text{M}-\text{H}]^-$ 172.0974), and taurine ($[\text{M}-\text{H}]^-$ 124.0068) duplicate standards in 50% aqueous acetonitrile at concentrations ranging from 0.19 to 100 μM . Theophylline (0.5 μM ; MH^+ 181.0726 m/z) and 4-nitrobenzoic acid (3 μM ; $[\text{M}-\text{H}]^-$ 166.0141) were included as internal standards. Quantification was accomplished using absolute peak area ratios (MH^+ , analyte/theophylline; $[\text{M}-\text{H}]^-$, analyte/4-nitrobenzoic acid) over standard concentrations for linear regression analysis. Calibration curves were linear for each analyte (r^2 , P) as follows: creatinine (0.96, <0.0001), β -thymidine (0.95, <0.001), *N*-hexanoylglycine (0.98, <0.0001), taurine (0.98, <0.0001). Analyte concentrations in mouse urine (diluted 5- to 400-fold in duplicate) were determined from the respective calibration curves. β -Thymidine, *N*-hexanoylglycine and taurine are expressed as $\mu\text{mol}/\text{mmol}$ creatinine (normalized). All samples and standards were run in duplicate, and the resultant concentrations were averaged. Analyte concentrations were tested for normal distribution by the skewness and kurtosis test. Mean concentrations of β -thymidine were tested for difference according to exposure status by a two-tailed t test assuming unequal variances ($\alpha = 0.05$; variance ratio test $P < 0.05$). Mean concentrations of *N*-hexanoylglycine in the 3-Gy experiment were tested for differences by the Mann-Whitney U test because at least one group in the comparison was not normally distributed. For the 8-Gy experiment, a t test assuming equal variances was used under the same parameters already mentioned. Mean taurine concentrations were tested using a t test assuming equal variances with the same parameters mentioned otherwise for the 3-Gy experiment and the Mann-Whitney U test for the 8-Gy experiment. Relative changes in analyte

concentrations were found by transforming the data to log base 2 followed by a two-tail *t* test. The mean differences and corresponding 95% confidence intervals were then used to calculate the relative change using 2^x , where *x* is the mean difference, lower value of the confidence interval, or upper value of the confidence interval. Potential confounding variables, namely body weight and urine sample volume, were also examined for exposure-specific differences. Mean sample volumes were compared by two-tailed *t* tests assuming equal variances as described above. For 3 Gy, mean body weights were compared by Mann-Whitney *U* test, whereas for 8 Gy the means were compared by a *t* test assuming unequal variances. All statistical analyses were performed using STATA (Stata Corp. LP, College Station, TX). Graphical presentations of data were prepared using Prism (GraphPad Software, Inc., San Diego, CA).

Gas Chromatography-Mass Spectrometry Analysis

Urine (100 μ l) was diluted with water (200 μ l), and standards were prepared in water (300 μ l). 4-Nitrobenzoic acid was used as internal standard with a final concentration of 5 μ M. Diluted urine samples and standards were applied to Waters Oasis HLB 1 ml (30 mg) SPE columns, washed with water (0.5 ml), and eluted with methanol (0.5 ml). The eluate was dried under nitrogen, resuspended in acetonitrile (50 μ l), and derivatized with BSFTA/10% TMCS (50 μ l). Samples were heated at 60°C for 30 min, then transferred to autoinjector vials for injection into the GCMS for analyte detection. GC/MS analysis was conducted on an Agilent GC 6890N/MS 5973N system using a J&W Scientific DB-225ms 30-m narrow-bore column. The initial oven temperature of 100°C was held for 2 min, then ramped at 10°C/min and held for 2 min at 220°C. The injector temperature was 200°C, and the interface temperature was 220°C. One microliter of sample was injected onto the column using helium as the carrier gas, held at a constant flow of 1 ml/min. The mass spectrometer was operated in scan mode (50–550 amu) with a solvent delay of 7 min. Citric acid tetra(trimethylsilyl) derivative, isocitric acid tetra(trimethylsilyl) derivative, and 4-nitrobenzoic acid mono(trimethylsilyl) derivative eluted at 11.4, 11.8 and 13.0 min, respectively. The data were quantified using extracted ions of *m/z* = 273 (citric acid), 245 (isocitric acid) and 224 (4-nitrobenzoic acid).

Bioinformatics

Gene Expression Dynamics Inspector (GEDi) (34,35) was used for the analysis and visualization of patterns in the MarkerLynx data matrices. The software package was developed for and has been applied in the past to the interpretation of gene expression data. GEDi creates intuitive visualizations of each sample based on the Self-Organizing Map (SOM) algorithm. However, it improves the interpretability of typical SOMs by rendering the output for each experimental sample as a two-dimensional heat-map-like mosaic of colored tiles. GEDi starts by training a conventional SOM to assign each ion to a mosaic tile in such a way that ions with similar patterns across the samples are placed in the same or nearby tiles. After that training, GEDi, unlike the conventional SOM algorithm, creates a series of coherent mosaic heat maps representing each sample's overall ion profile. The GEDi analysis here used Pearson's correlation as the similarity metric in training of the SOM. In addition, to identify the common expression patterns within each dose group, GEDi was used to compute average mosaics.

RESULTS

Influence of Cage Stress on the Mouse Urinary Metabolome

Preliminary investigations determined that the handling and caging of all mice influenced their urinary metabolome (data not shown). In particular, PCA revealed the elevation of a particular urinary constituent that at first was believed to be due to the stress of housing of individual mice in metabolic cages with metallic mesh floors. This urinary constituent was elevated up

to twofold during the second day in the metabolic cage environment but dropped back to baseline levels during a fourth day in the metabolic cage. The ion in question had a retention time of 0.40 min and $m/z = 144.1020$ in positive ion mode, corresponding to an empirical formula for the protonated molecular ion of $C_7H_{14}NO_2$ with 3.5 ppm error. Both co-chromatography with an authentic standard and tandem MS fragmentation spectra established that this ion is derived from stachydrine (proline betaine, *N*-methylproline) in urine. Stachydrine is found in alfalfa, where it is synthesized from ornithine (36) and therefore is likely a constituent of the laboratory animal chow. Extraction of the NIH31 diet with water/ acetonitrile mixtures confirmed the presence of stachydrine in the diet. Therefore, we propose that the increased urinary excretion of stachydrine during the first three occasions that the mice are placed in metabolic cages is a simple reflection of increased feeding and is not attributable to a psychological stress response. Stachydrine is reported to be biotransformed in rats to various oxidized and conjugated urinary metabolites (37), and presumably the same is true in mice. Accordingly, in all experiments, mice were first acclimated to the metabolic cages on three prior occasions as shown in Fig. 1 to minimize this dietary/stress effect on the urinary metabolome.

Metabolomic Analysis of Mouse Urine after 3 Gy γ Irradiation

Mice were irradiated with 3 Gy ($n = 12$) or sham irradiated ($n = 12$), and their urine was collected in metabolic cages for 0–24 h. Irradiated mice appeared to have smaller urine volumes (0.84 ± 0.62 ml, mean $\pm$ SD) than sham-irradiated mice (1.20 ± 0.79 ml), but this difference was not statistically significant. UPLC-TOFMS analysis of urine revealed a large data matrix containing approximately 6,000 negative ions per mouse urine sample, which was subjected to both PCA and OPLS multivariate data analyses. Unsupervised PCA did not give a good clustering of the data sets for sham-irradiated and irradiated animals (data not shown). Therefore, supervised OPLS analysis was performed whereby the data were classified as either irradiated or sham. Figure 2A shows an OPLS scores plot for the 3-Gy experiment, depicting a clear separation between mice that were irradiated and those that were sham irradiated. Figure 2C shows a scatter S-plot from the OPLS analysis of the 3-Gy data. The 22 most outlying ions have been annotated 1 to 22.

Identification of Mouse Urinary Biomarkers after 3 Gy γ Irradiation

Biomarker 1 (and its + 1 isotope, biomarker 6), from the OPLS scatter S-plot in Fig. 2C, with a $[M-H]^- = 230.996$, gave an empirical formula of $C_8H_7O_6S^-$ with a mass error of 1.3 ppm. Treatment of urine with arylsulfatase (Type H-1 from *Helix pomatia*) caused this ion to disappear, confirming that it derived from a sulfate conjugate. The metabolite that is conjugated with sulfate would therefore have an empirical formula of $C_8H_8O_3$, for which there are 14 possible carboxylic acid candidates, namely, 2-, 3- and 4-hydroxyphenylacetic acid, mandelic acid, 3-, 4-, 5- and 6-methylsalicylic acid, 3-hydroxy-2-methyl-, 3-hydroxy-4-methyl-, 3-hydroxy-5-methyl-, 5-hydroxy-2-methyl-, 4-hydroxy-2-methyl-, and 4-hydroxy-3-methylbenzoic acid. In addition, two aldehydes with the same empirical formula might also be conjugated with sulfate and found in urine, 3-hydroxy-4-methoxybenzaldehyde (isovanillin) and 4-hydroxy-3-methoxybenzaldehyde (vanillin). However, it has long been established that these aldehydes are largely oxidized to their respective benzoic acids prior to urinary excretion in laboratory animals (38,39). Thus the investigation of the sulfate metabolite was restricted to the 14 aforementioned organic acids. Details of the mass fragmentation of deprotonated molecular ions ($[M-H]^-$) of each of the sulfated aromatic acids are shown in Table 1. Inspection of the UPLC retention times and MS/MS fragmentation patterns readily revealed that the urinary sulfate with $[M-H]^- = 230.9962$ could not be a phenylacetic acid or salicylic acid derivative. However, the sulfate of 3-hydroxy-2-methylbenzoic acid co-chromatographed with the urinary peak and had an identical fragmentation pattern (Fig. 3). The metabolic origin of 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate is not known.

Co-chromatography and tandem MS with authentic standards demonstrated unequivocally that the identity of biomarker 2 (Table 2), with a $[M-H]^- = 172.0985\ m/z$ ($C_8H_{14}NO_3^-$, mass error = 6.4 ppm) and retention time of 3.66 min, was *N*-hexanoylglycine. There were no other high-ranking ions derived from this ion (isotopes, in-source fragments, adducts, dimers).

The identity of biomarker 4, with a $[M-H]^- = 191.0207\ m/z$ ($C_6H_7O_7^-$, mass error = 7.9 ppm) and retention time of 0.43 min, was established as either citric acid or isocitric acid, since both isomers co-chromatographed in the UPLC system. Moreover, biomarker 5, with a $[M-H]^- = 111.008\ m/z$, was determined to be an in-source fragment ion of either citric acid or isocitric acid. The identity of this biomarker 4 was determined by GCMS, which, unlike UPLC-TOFMS, was able to separate citric acid from isocitric acid. The results showed that neither citric acid nor isocitric acid was statistically significantly elevated by irradiation with 3 Gy.

The identity of biomarker 8, with a $[M-H]^- = 241.0820\ m/z$ ($C_{10}H_{13}N_2O_5^-$, mass error = 1.7 ppm) and retention time of 1.90 min, was thymidine. This was confirmed by tandem MS experiments. However, there are two epimers of thymidine, β -thymidine, the nucleoside present in DNA, and α -thymidine, which can be formed in DNA *in situ* by oxidative stress in the nucleus and removed by nucleotide excision repair (40). These did not resolve by UPLC, with α - and β -thymidine having retention times of 1.94 and 1.92 min, respectively. Moreover, both epimers had identical tandem MS spectra, with ions of nominal m/z (% abundance) of 241 (100) and 151 (10). Therefore, a longer (150 mm) UPLC column was used for their analysis, which resolved β -thymidine (3.63 min) from α -thymidine (3.56 min) with a 90% peak-to-peak valley. Additionally, when β -thymidine was added to urine, the extracted ion chromatogram (m/z 241.0824) showed two peaks. When urine was spiked with β -thymidine, only one peak was observed. We concluded that biomarker 8 was β -thymidine. There were no other high-ranking ions (isotopes, in-source fragments, adducts, dimers) derived from this ion.

Biomarker 11 had a $[M-H]^- = 417.1143\ m/z$ ($C_{10}H_{13}N_2O_5^-$, mass error = 1.7 ppm), which would match to a glucuronide of thymidine. However, this biomarker was of low abundance and experiments with β -glucuronidase hydrolysis were inconclusive. Moreover, a glucuronide of thymidine has never been reported. The identity of this biomarker was not pursued further.

Thus, three biomarkers for γ irradiation of mice with 3 Gy were unequivocally identified as 3-hydroxy-2-methyl-benzoic acid 3-*O*-sulfate, *N*-hexanoylglycine and β -thymidine.

Metabolomic Analysis of Mouse Urine after 8 Gy γ Irradiation

When mice were irradiated with 8 Gy ($n = 12$) or were sham irradiated ($n = 12$), urine volumes appeared to be smaller in the irradiated mice (0.77 ± 0.58) than the sham-irradiated mice (1.03 ± 0.46), but this difference was not statistically significant. UPLC-TOFMS analysis produced a data matrix of approximately 6,000 ions that was analyzed by PCA, and this also did not give a good clustering of irradiated and sham-irradiated mice (data not shown). However, OPLS analysis showed a clear separation and clustering of the groups in the plot of the scores (Fig. 2B). Fig. 2D displays the scatter S-plot from the OPLS analysis of the 8-Gy data, with the top 22 outlying ions annotated 1 to 22.

Identification of Mouse Urinary Biomarkers after 8 Gy γ Irradiation

Of these 22 ions, biomarker 1 (Table 3), with a $[M-H]^- = 172.0985\ m/z$ ($C_8H_{14}NO_3^-$, mass error = 6.4 ppm) and retention time of 3.66 min, was *N*-hexanoylglycine. The identity of biomarker 4, with a $[M-H]^- = 191.0207\ m/z$ ($C_6H_7O_7^-$, mass error = 7.9 ppm) and retention time of 0.43 min, could be established only as either citric acid or isocitric acid. GC/MS analysis (see above) showed that neither citric acid nor isocitric acid was statistically significantly elevated in mouse urine after irradiation with 8 Gy. The identity of biomarker 12, with a $[M-$

$\text{H}]^- = 124.0080 \text{ } m/z$ ($\text{C}_2\text{H}_6\text{NO}_3$, mass error = 9.7 ppm) and retention time of 0.29 min, was taurine, as demonstrated by co-chromatography with authentic standard and identical tandem MS fragmentation. The identity of biomarker 13, with a $[\text{M}-\text{H}]^- = 241.0820 \text{ } m/z$ ($\text{C}_{10}\text{H}_{13}\text{N}_2\text{O}_5$, mass error = 1.7 ppm) and retention time of 1.90 min, was β -thymidine. Thus, three biomarkers for γ irradiation of mice with 8 Gy were unequivocally identified as *N*-hexanoylglycine, taurine and β -thymidine.

Quantification of Urinary Biomarkers after 0, 3 and 8 Gy γ Irradiation

The concentration of the discovered biomarkers β -thymidine, *N*-hexanoylglycine and taurine as well as creatinine were all measured in each urine sample after construction of calibration curves using theophylline (ESI + mode) and 4-nitrobenzoic acid (ESI – mode) as internal standards. Because no authentic sample of sufficient quantity was available for 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate, comparisons of excretion of this biomarker after doses of 0, 3 and 8 Gy were made on the basis of relative peak area, normalized to creatinine. Urinary creatinine concentration varied widely between groups of mice. Specifically, the 3-Gy-irradiated and sham-irradiated controls had creatinine concentrations of $3.26 \pm 0.91 \text{ mM}$ and $2.83 \pm 0.56 \text{ mM}$, respectively, which were not statistically significantly different. The 8-Gy-irradiated and sham-irradiated controls had urinary creatinine concentrations of $1.95 \pm 0.51 \text{ mM}$ and $1.38 \pm 0.33 \text{ mM}$, respectively, which were statistically significantly different ($P = 0.003$). However, when these concentrations were multiplied by urine volumes, the sham-irradiated mice excreted a mean of $1.32 \pm 0.52 \text{ }\mu\text{mol}$ creatinine in 0–24 h and the 8-Gy-irradiated mice excreted a mean of $1.30 \pm 0.75 \text{ }\mu\text{mol}$ creatinine in 0–24 h, values that were not statistically significantly different. Thus, the irradiated mice simply produced a small volume of more concentrated urine and 8 Gy radiation had no apparent effect on the total production and excretion of creatinine. Consequently, biomarkers were then expressed as $\mu\text{mol}/\text{mmol}$ creatinine, a variable largely unaffected by urine volume. The urinary creatinine concentrations are displayed in Fig. 4A.

Urinary 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate was elevated 2.5-fold after irradiation with 3 Gy but not after 8 Gy (Fig. 4B). *N*-Hexanoylglycine was elevated 40–80% after γ irradiation (Fig. 4C), from 358 ± 176 to $643 \pm 259 \text{ }\mu\text{mol}/\text{mmol}$ creatinine ($P = 0.002$) for 3 Gy and from 556 ± 198 to $802 \pm 117 \text{ }\mu\text{mol}/\text{mmol}$ creatinine for 8 Gy. β -Thymidine was elevated six- to sevenfold after γ irradiation (Fig. 4D), from 9.97 ± 4.76 to $67.7 \pm 17.4 \text{ }\mu\text{mol}/\text{mmol}$ creatinine ($P < 0.001$) for 3 Gy irradiation and from 5.48 ± 1.46 to $35.6 \pm 8.84 \text{ }\mu\text{mol}/\text{mmol}$ creatinine ($P < 0.001$) for 8 Gy. Urinary taurine was elevated only after 8 Gy (Fig. 4E); the values for sham irradiation and 3 Gy were 7.57 ± 2.36 and $8.52 \pm 1.90 \text{ mmol}/\text{mmol}$ creatinine, respectively. These very high excretion values were not statistically significantly different. After 8 Gy irradiation, the urinary taurine excretion increased 20% from 7.37 ± 1.25 to $8.91 \pm 1.29 \text{ mmol}/\text{mmol}$ creatinine ($P = 0.004$). None of the differences in urine volume, creatinine or biomarker excretion could be explained on the basis of the differences in body weights (30.9 ± 1.7 , 30.1 ± 2.2 , 30.1 ± 1.1 , and $31.1 \pm 2.3 \text{ g}$ for the 3-Gy sham, 3-Gy irradiated, 8-Gy sham, and 8-Gy irradiated groups, respectively), which were not statistically significantly different from each other.

Global Urinary Metabolome Changes in Response to γ Irradiation Display a Dose–Response Relationship as Determined by GEDI Self-Organizing Maps

A holistic view of the mouse urinary metabolome was made using GEDI software, which was originally designed for analyzing gene expression profiles. This bioinformatics process facilitates visualization of regions of the urinary metabolome that increased and decreased in concentration in response to γ -radiation exposure. Figure 5 shows a series of self-organizing maps for the average urinary metabolomes of groups of mice that had been irradiated with 0, 6, 7, 8 or 11 Gy in the first 24 h after exposure. A clear dose–response relationship exists for

both a group of negative ions in the bottom left-hand corner that decrease in abundance with radiation dose (panel B) and a group of negative ions that increase in abundance with increasing radiation dose (panel C). These submatrices of 3×3 cells contain approximately 7.5% of all the ions that comprise the urinary metabolome that is displayed in the 13×11 -cell matrix of the self-organizing maps. Moreover, two of the known elevated biomarkers, β -thymidine and *N*-hexanoylglycine, fall in the bottom right-hand submatrix of 3×3 cells (panel C). Similar map areas also decreased and increased, respectively, in relative abundance when positive ions were analyzed (panel D). It is important to note that the positive ions represented in Fig. 5D are distinct urine metabolites from the negative ions shown in Fig. 5A–C, with perhaps only a little overlap with ions that can appear in both negative and positive ionization MS. Together, these results demonstrate, for the first time, a dose–response relationship between γ radiation and biomarkers in the mouse urinary metabolome.

Urinary Metabolomic Phenotypes for the Detection of γ -Radiation Exposure

The combination of pairs of urinary biomarkers, specifically, *N*-hexanoylglycine and taurine, *N*-hexanoylglycine and β -thymidine, together with taurine and β -thymidine, were evaluated for their ability to define a urinary metabolomic phenotype that was diagnostic of γ -radiation exposure. Figure 6 displays these phenotypes for mice exposed to 3 Gy and 8 Gy relative to sham-irradiated (control) animals. Plots of *N*-hexanoylglycine as a function of taurine were uninformative for both 3 Gy (Fig. 6A) and 8 Gy (Fig. 6B). However, plots of *N*-hexanoylglycine as a function of β -thymidine segregated into two phenotypes for 0 compared to 3 Gy (Fig. 6C) and 0 compared to 8 Gy (Fig. 6D). Additionally, plots of taurine as a function of β -thymidine segregated into two phenotypes for 0 compared to 3 Gy (Fig. 6E) and 0 compared to 8 Gy (Fig. 6F). In these models, a 3-Gy exposure was not distinguishable from an 8-Gy exposure.

DISCUSSION

Analysis by UPLC-TOFMS of 24-h urine samples collected immediately after exposure of mice to 3 and 8 Gy γ radiation produced data matrices of m/z compared to retention time compared to normalized ion intensity that, when subjected to multivariate data analysis by OPLS, revealed distinct metabolomic phenotypes for each dose and for sham-irradiated animals (Fig. 2). From the top 22 ions contributing to this clustering and inter-phenotype separation, a number of urinary biomarkers were unequivocally identified using tandem mass spectrometric comparison with authentic standards. A novel biological molecule, 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate was a biomarker of 3 Gy but not 8 Gy. *N*-Hexanoylglycine and β -thymidine were biomarkers of exposure to both 3 and 8 Gy (Tables 2 and 3). Taurine was a biomarker of 8 Gy only. In general, the increase in biomarker excretion in urine of exposed over sham-irradiated animals was 1.2- to 2.5-fold. The one exception was β -thymidine, in which case the presence in urine was elevated six- to seven-fold after γ irradiation. In addition, several in-source fragment and isotope ions were identified. Finally, we demonstrate a clear dose–response relationship in the global view of the urine metabolite profile visualized using GEDI.

The chemical identities of four markers have been elucidated, of which two, β -thymidine and *N*-hexanoylglycine, are validated and quantified across the two experiments. Because these ions are elevated in urine from exposed animals at both doses, we conclude that these are specific biomarkers of radiation exposure. In addition, we found 3-hydroxy-2-methylbenzoic acid *O*-sulfate statistically significantly elevated in the urine of animals exposed to 3 Gy but not 8 Gy compared with controls. We also observed that taurine is statistically significantly elevated in the urine of mice exposed to 8 Gy but not 3 Gy compared with controls. The point estimate of mean taurine level in the urine from mice exposed to 3 Gy is elevated over that of the controls, albeit not significantly. This is suggestive of a dose–response relationship that

needs to be examined further at doses intermediate between 3 and 8 Gy. In addition, there are several other ions among the 22 ions highlighted in each experiment that are not common to both experiments.

Although it was not possible to identify unambiguously all urinary constituents that were elevated after γ irradiation of mice, a bioinformatic technique was used that demonstrated that a large number of urinary constituents co-varied across the sample set with the aforementioned biomarkers. In other words, the phenotypes for 3 Gy and 8 Gy that were seen as distinct clusters in the plots of OPLS scores (Fig. 2A and B, respectively) arose due to numerous differences in urinary constituents between the irradiated and sham-irradiated animals. This can be seen from the GEDI self-organizing maps, where groups of nine interconnected tiles, which represented hundreds of both negative (Fig. 5A) and positive (Fig. 5D) ions, increased (Fig. 5C) in intensity in a dose-dependent manner, while others decreased (Fig. 5B), also in a clear dose-dependent fashion. This is an important proof of principle of radiation metabolomics and is also the first time that GEDI self-organizing maps have been used to analyze and display global *in vivo* metabolomic data. We take these observations to be a sign of the existence of a rich source of additional biomarkers of radiation exposure.

Using pairs of biomarkers (Fig. 6), it may ultimately be possible to predict whether a mouse has been exposed to γ radiation and perhaps also the general dose range. This approach, refined by the future addition of biomarkers, is expected to lead to a metabolomics-based protocol for non-invasive radiation biodosimetry in humans. Considerably more work needs to be done, and a re-evaluation of the published literature appears to be in order. To this end, Table 4 lists the small molecule biomarkers of ionizing radiation exposure reported for both laboratory animals and humans, together with the calculated m/z values of their protonated and deprotonated molecular ions. It is of note that none of the validated radiation biomarkers that are reported here have been reported previously. The published biomarkers fall into the classes of neurotransmitter metabolites, excised DNA adducts, reactive oxygen products, and general metabolic intermediates. A search for these positive and negative ions in our data set established that none of these published biomarkers were statistically significantly elevated within 1 day after exposure of mice to 3 and 8 Gy γ radiation except for 192.027, citric acid. In this case, we attempted to but could not determine whether citric acid or isocitric acid or both were detected. Thus the identity of the $[M-H]^-$ ion of m/z 191.0192 elevated in urine from radiation-exposed mice remains to be elucidated. Future research will determine whether any of the ions listed in Table 4 are elevated at later times.

The question arises as to the metabolic origins of the novel radiation biomarkers reported here. The most dramatic change after irradiation was in β -thymidine (Fig. 4D), which may reflect increased synthesis, decreased use or elevated renal tubular outward transport. It is also possible that the products of oxidative DNA damage, thymine glycol and thymidine glycol (19,20), might be metabolically reconverted to thymidine, although this is known not to occur in *E. coli* (41) and is therefore unlikely. Interestingly, when [3H]thymidine was administered intravenously to patients, the radioactivity found in urine was approximately 100 times that in plasma (42), suggesting that extracellular thymidine is rapidly excreted into urine. The elevated thymidine excretion reported here is therefore a potential marker of increased DNA breakdown and cell turnover due to γ radiation.

Elevated urinary excretion of *N*-hexanoylglycine is usually interpreted as a sign of impaired medium-chain fatty acid metabolism, that is, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, although this is usually accompanied by the excretion of dicarboxylic acids and free fatty acids (43). These additional metabolic signs did not appear in our metabolomic analysis, suggesting that the elevated appearance of *N*-hexanoylglycine in urine may not be a result of an effect of γ radiation on hepatic mitochondrial MCAD. We have

recently reported that urinary excretion of *N*-hexanoylglycine is reduced 20-fold after activation of the nuclear receptor PPAR α in mice (44) and it has been reported that PPAR α appears to play a role in the response of mice to 10 Gy γ radiation (45). How these two lines of evidence are related is currently unknown.

Elevated taurine excretion in urine was first reported to be associated with carbon tetrachloride liver damage (46), but metabolomic studies have since characterized it as a general urinary marker of hepatotoxicity (47,48). Since taurine is an end product of cysteine catabolism, it has been proposed that urinary excretion of taurine represents evidence of increased cysteine use in the liver in response to toxic injury (48). The elevation in urinary excretion of taurine reported here is modest and occurred only after the 8-Gy dose (Fig. 4E). Increased hepatic or renal cysteine/glutathione turnover is one possible explanation.

The elevation of urinary 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate after 3 Gy but not 8 Gy is without precedent. All possible isomers of this compound were synthesized *in situ* and evaluated by tandem mass spectrometry, and this organic acid sulfate gave a perfect match to the urinary peak by both retention time and mass fragmentography (Fig. 3). To our knowledge, the parent 3-hydroxy-2-methylbenzoic acid has not been described before in biological systems. Isomeric hydroxymethylbenzoic acids, however, are known bacterial metabolites and may arise from the gut flora. Further characterization of this biomarker falls beyond the scope of this report.

In summary, we report here a metabolomic investigation of mice after γ irradiation with 3 and 8 Gy. OPLS analysis of mass spectrometric data matrices revealed novel biomarkers that were statistically significantly elevated in urine. GEDI self-organizing maps demonstrate the existence of dose-dependent excretion of a subset of global urinary biomarkers. These data will be useful to help design strategies for noninvasive radiation biodosimetry through metabolomics in human populations.

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FIG. 1.

Urine sample collection protocol for radiation metabolomics. Two groups of mice were used to identify γ -radiation-specific changes in urine metabolites, exposure (A, $h\nu$), and sham control handling (B, Sham). Urine collections were made in alternating periods whereby the mice spent 24 h individually in metabolic cages (—) and then 24 h in their holding room cages (.....) with littermates. Three pre-exposure collections were taken to allow the animals to adapt to the handling and new environments. Urine was then collected for 24 h immediately after radiation and sham exposures.

FIG. 2.

OPLS scores and loadings plots for urine samples from control and irradiated mice. Component 1 (abscissa) scores for urine collected over the first 24 h after exposure from control ($\circ$) and irradiated ($\bullet$) mice for doses of 3 (panel A) and 8 Gy (panel B) show class separation based on exposure status. Ions are found to be elevated in urine from mice exposed to 3 Gy (panel C) and 8 Gy (panel D) compared to the respective controls. Loadings reveal ions in a spatial relationship to the class separation. At each dose, the best 22 ions that serve as candidate biomarkers of radiation exposure are indicated (numbered; also see insets).

FIG. 3.

Determination of the chemical structure of the 3-Gy biomarker 1 by tandem mass spectrometry. Top panel shows the negative ion MS/MS fragmentation of synthetic 3-hydroxy-2-methylbenzoic acid 3-O-sulfate, which eluted at 2.1 min. Bottom panel shows the negative ion MS/MS fragmentation of a peak in mouse urine, also eluting at 2.1 min.

FIG. 4.

Relative increases in urinary creatinine and biomarkers at 3 and 8 Gy over those in sham-irradiated animals. Panel A: Creatinine; panel B: 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate; panel C: *N*-hexanoylglycine; panel D: β-thymidine; panel E: taurine. All data for the quantified biomarkers (panels C–E) were normalized to the creatinine concentration (μmol/mmol creatinine, see text). Data for 3-hydroxy-2-methylbenzoic acid 3-*O*-sulfate (panel B) are based on relative peak areas and then normalized to creatinine (see text). Asterisks indicate statistically significant elevations over controls (see text). Data are means with 95% confidence intervals.

FIG. 5.

Dose response of the mouse urinary metabolome to γ radiation. Panel A: Self-organizing maps that give a holistic view of the urinary metabolome in a 13×11 matrix (average of 42 ions per cell) constructed using GEDI software. Data used comprise approximately 6,000 negative ions (ESI – mode). Panel B: Sum total of relative intensities of negative ions in a 3×3 matrix in bottom left-hand corner of the maps, with background subtraction (values for 11 Gy). There is a clear decrease in radiation dose response in biomarkers in this region of the metabolome. Panel C: Sum total of relative intensities of negative ions in a 3×3 matrix in bottom right-hand corner of the maps, with background subtraction (values for 0 Gy). There is a clear increase in radiation dose response in biomarkers in this region of the metabolome. Panel D: Self-organizing maps that give a holistic view of the urinary metabolome in a 13×11 matrix (average of 42 ions per cell) constructed using GEDI software. Data used comprise approximately 6,000 positive ions (ESI + mode). Note that the areas of the positive ion maps that increase and decrease with radiation dose correspond to the areas of the negative ion maps that increase and decrease with radiation dose (panel A).

FIG. 6.

Predictive ability of biomarker combinations for ionizing radiation exposure in mice. Panel A: *N*-Hexanoylglycine and taurine (0 compared to 3 Gy). Panel B: *N*-Hexanoylglycine and taurine (0 compared to 8 Gy). Panel C: *N*-Hexanoylglycine and β-thymidine (0 compared to 3 Gy). Panel D: *N*-Hexanoylglycine and taurine (0 compared to 8 Gy). Panel E: Taurine and β-thymidine (0 compared to 3 Gy). Panel F: Taurine and β-thymidine (0 compared to 8 Gy).

TABLE 1

Organic Acids Sulfated *In Situ* to Identify the Urinary Negative Ion of 230.996 *m/z*

Isomer	Structure	Retention time of sulfate (min)	Principal ions of sulfate [MS/MS] (abundance, %)
Urine peak	—	2.12	230.995 (60), 187.004 (3), 151.038 (100), 107.050(50)
2-Hydroxyphenyl-acetic acid		2.09	230.997(100), 187.005 (70), 152.918 (30), 151.038 (20), 107.049 (80)
3-Hydroxyphenyl-acetic acid		2.09	230.997 (100), 187.005 (90), 152.917 (40), 107.049 (20)
4-Hydroxyphenyl-acetic acid		1.84	230.995 (100), 187.008 (30), 152.916 (20), 147.049 (0)
Mandelic acid		1.96	230.995 (25), 151.038 (35), 96.959 (100)
3-Methylsalicylic acid		2.00	230.997 (25), 151.038 (35), 96.959 (100)
4-Methylsalicylic acid		1.85	230.997 (15), 212.986(100), 184.991 (25), 156.996 (10)
5-Methylsalicylic acid		1.94	230.991 (45), 187.004 (15), 152.917 (100), 96.963 (75)
6-Methylsalicylic acid		1.90	230.995 (100), 187.008 (30), 151.040 (5), 107.049(50), 79.959 (40)
3-Hydroxy-2-methyl-benzoic acid		2.11	230.995(50), 187.004 (3), 151.038 (100), 107.050(70)
3-Hydroxy-4-methyl-benzoic acid		2.78	230.993 (40), 151.037 (100), 107.049 (65)
3-Hydroxy-5-methyl-benzoic acid		2.93	230.995 (100), 199.808 (35), 195.807 (70), 195.810(60), 160.841 (50), 151.039 (10), 121.028 (40)
5-Hydroxy-2-methyl-benzoic acid		2.71	230.997(60), 151.040(100), 107.050 (90)

Isomer	Structure	Retention time of sulfate (min)	Principal ions of sulfate [MS/MS] (abundance, %)
4-Hydroxy-2-methylbenzoic acid		2.26	230.996(100), 187.007 (40), 152.915 (15), 151.037 (10), 107.051 (45)
4-Hydroxy-3-methylbenzoic acid		2.39	231.000(100), 152.909(60), 151.032 (15), 96.961 (40)

TABLE 2
Identification of Mouse Urinary Biomarkers after 3 Gy γ Radiation

Marker no.	Retention Time (min)	Mass		ppm error	Ion formula	Identity
		Found	Calculated			
1	2.29	230.9962	230.9963	1.3	$C_8H_7O_6S^-$	3-Hydroxy-2-methylbenzoic acid <i>O</i> -sulfate
2	3.65	172.0950	172.0974	13.9	$C_8H_{14}NO_3^-$	<i>N</i> -Hexanoylglycine
4	0.43	191.0192	191.0192	0.0	$C_7H_7O_7^-$	Citric acid and/or isocitric acid
5	0.44	111.0080	—	—	—	In-source fragment of no. 4
6	2.28	231.9980	—	—	—	Isotope (+1) of no. 1
8	1.90	241.0824	241.0824	0.0	$C_{10}H_{13}N_2O_5^-$	β -Thymidine
11	1.82	417.1143	417.1145	0.4	$C_{16}H_{21}N_2O_{11}^-$	Putative thymidine 5'- β -D-glucuronide
13	1.74	267.0741	267.0729	4.1	$C_{10}H_{11}N_4O_5^-$	Putative inosine
18	4.41	93.0341	—	—	—	In-source fragment

TABLE 3
Identification of Mouse Urinary Biomarkers after 8 Gy γ Radiation

Marker no.	Retention time (min)	Mass		ppm error	Ion formula	Identity
		Found	Calculated			
1	3.66	172.0974	172.0974	0.0	$C_8H_{16}NO_3^-$	<i>N</i> -Hexanoylglycine
4	0.43	191.0191	191.0192	0.5	$C_7H_7O_7^-$	Citric acid and/or isocitric acid
6	3.66	74.0294	—	—	—	In-source fragment of no. 1
8	0.42	111.0112	—	—	—	In-source fragment of no. 4
9	3.64	173.1000	—	—	—	Isotope (+1) of no. 1
10	0.37	85.0330	—	—	—	In-source fragment of no. 4
11	0.29	124.0092	124.0068	24.2	$C_2H_6NO_3S^-$	Taurine
12	1.90	241.0836	241.0824	3.7	$C_{10}H_{13}N_2O_5^-$	β -Thymidine
19	0.37	117.0220	—	—	—	In-source fragment of no. 4

TABLE 4

Historical Biomarkers of Radiation Exposure

Chemical name	Empirical formula	Mono-isotopic mass (M)	MH+	[M-H] ⁻	Ref(s).	Note
Glycine	C ₂ H ₃ NO ₂	75.032	76.0399	74.0242	24	1
Thymine glycol	C ₅ H ₈ N ₂ O ₄	160.0484	161.0562	159.0406	19,20	1
Homovanillic acid	C ₉ H ₁₀ O ₄	182.0579	183.0657	181.0501	18	1
4-Hydroxy-3-methoxyphenylglycol	C ₉ H ₁₂ O ₄	184.0736	185.0814	183.0657	18	1
Metanephrene	C ₁₀ H ₁₅ NO ₃	197.1052	198.113	196.0974	18	1
4-Hydroxy-3-methoxymandelic acid	C ₉ H ₁₀ O ₅	198.0528	199.0606	197.045	18	1
Indoxyl sulfate	C ₈ H ₇ NO ₄ S	213.0096	214.0174	212.0018	15	1
Thymidine glycol	C ₁₀ H ₁₆ N ₂ O ₇	276.0958	277.1036	275.0879	19	1
8-Isoprostaglandin F _{2a}	C ₂₀ H ₃₄ O ₅	354.2406	355.2484	353.2328	22-23	1
Thromboxane B ₂	C ₂₀ H ₃₄ O ₆	370.2355	371.2434	369.2277	21	1
8-Hydroxyguanine	C ₅ H ₅ N ₅ O ₂	167.0443	168.0521	166.0365	20	2
Creatinine	C ₄ H ₇ N ₃ O	113.0589	114.0667	112.0511	24	3
Hippuric acid	C ₉ H ₉ NO ₃	179.0582	180.0661	178.0504	24	3
Normetanephrene	C ₉ H ₁₃ NO ₃	183.0895	184.0974	182.0817	18	3
5-Hydroxyindoleacetic acid	C ₁₀ H ₉ NO ₃	191.0582	192.0661	190.0504	13-17	3
Citric acid	C ₆ H ₈ O ₇	192.027	193.0348	191.0192	24	4

Notes. ¹[M-H]⁻ (± 10 ppm) either not detected or not statistically significantly different ($P \geq 0.05$) according to exposure status; ²[M-H]⁻ (± 10 ppm) elevated ($P < 0.05$) in urine from mice exposed to 3 Gy; ³[M-H]⁻ (± 10 ppm) elevated ($P < 0.05$) in urine from mice exposed to 8 Gy; ⁴[M-H]⁻ (± 10 ppm) elevated ($P < 0.05$) in urine from mice both exposed to 3 and 8 Gy. All comparisons made with mean creatinine-normalized relative concentrations by t test with $\alpha = 0.05$.