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Differential Mobility Spectrometry-Mass Spectrometry (DMS-MS) in Radiation Biodosimetry: Rapid and High-Throughput Quantitation of Multiple Radiation Biomarkers in Nonhuman Primate Urine

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Abstract

High-throughput methods to assess radiation exposure are a priority due to concerns that include nuclear power accidents, the spread of nuclear weapon capability, and the risk of terrorist attacks. Metabolomics, the assessment of small molecules in an easily accessible sample, is the most recent method to be applied for the identification of biomarkers of the biological radiation response with a useful dose-response profile. Profiling for biomarker identification is frequently done using a LC-MS platform which has limited throughput due to the time-consuming nature of chromatography. We present here a chromatography-free simplified method for quantitative analysis of seven metabolites in urine with radiation dose-response using urine samples provided from the Pannkuk et al. (2015) study of long-term (7-day) radiation response in non-human primates (NHP). The stable-isotope-dilution (SID) analytical method consists of sample preparation by strong cation exchange-solid phase extraction (SCX-SPE) to remove interferences and concentrate the metabolites of interest, followed by differential mobility spectrometry (DMS) ion filtration to select the ion of interest and reduce chemical background, followed by mass spectrometry (overall SID-SPE-DMS-MS). Since no chromatography is used, calibration curves were prepared rapidly, in under 2 hours (including SPE) for six simultaneously analyzed radiation biomarkers. The seventh, creatinine, was measured separately after 2500X dilution. Creatinine plays a dual role, measuring kidney glomerular filtration rate (GFR), and indicating kidney damage at high doses. The current quantitative method using SID-SPE-DMS-MS provides

throughput which is 7.5 to 30 times higher than that of LC-MS and provides a path to pre-clinical radiation dose estimation.

Graphical abstract

Keywords

Biomarkers; Radiation exposure; Nonhuman primate; metabolomics; Quantitation; Differential mobility spectrometry; Field asymmetric waveform ion mobility spectrometry; DMS-MS; FAIMS-MS

Introduction

In the most recent report by the International Atomic Energy Agency (IAEA), 234 events were reported for 2016 that were involving, or suspected to involve, ionizing radiation, 31 requiring responses by the IAEA Incident and Emergency Centre (IEC) [1]. Some types of incidents would lead to large numbers of subjects requiring prompt assessment in a rapid manner for exposure to ionizing radiation (IR), making the development of a rapid, field-deployable method to assess radiation exposure increasingly important. Although exposure to radiation can result in acute radiation syndrome (ARS) and more serious symptoms or death, a number of radio-protective and radio-mitigative treatments are effective if applied in a timely fashion after the dose has been estimated to require it [2]. For determining the dose, the dicentric assay (DCA) is the only method approved by the Food and Drug Administration (FDA) for radiation assessment, however, this method is labor intensive, time-consuming, and laboratory-limited. It is therefore important and necessary to develop new technologies that are simple, rapid and widely usable.

Our method development employs samples and biomarker identifications obtained in a 2015 global profiling urine metabolomics study by Pannkuk et al. [3] that was focused on the long-term (7-day) radiation response in non-human primates (NHP). Detailed animal handling protocols and descriptions of the discovery experiments appear in that reference [3]. The samples used here are described in the Experimental section.

Global profiling metabolomics, the approach of discovering significant metabolites associated with radiation exposure, have identified key radiation biomarkers in urine and blood that may be used to detect differences between dose levels, dose rate, dose quality, and external or internal exposure in various animal models and patients, as recently reviewed [4].

Ionizing radiation has been shown to dysregulate the normal metabolism and thus changes the levels of the metabolites. Studies that have been conducted include several in rodent models to identify metabolic differences associated with external exposure [5–12], effects of dose rate [5], specificity [13], internal exposures [14–17], and dose quality [13]. Metabolic signatures have also been developed for nonhuman primates (NHPs) exposed to external total body irradiation (TBI) [3, 18–21]. Human populations who have received total body irradiation (TBI) as a medical treatment [22] have also been studied. Identification and quantification of such variations of the metabolites are being investigated for application to biodosimetry and screening of exposed populations by targeted metabolomics.

Targeted metabolomics applies the profiling results by focusing on a select panel of metabolites that can be quantified in small volumes of biological samples. Such targeted approaches are already in use in clinical laboratories nationwide with applications in toxicology and endocrinology (e.g. Vitamin D quantitation [23]) and expanding in newborn screening for inborn errors of metabolism [9, 11, 24].

LC-MS has long served as the gold standard for metabolomics studies in radiation exposure due to its compatibility with different classes of metabolites using different separation modes such as normal phase, reversed phase, ion exchange, etc., for simultaneous monitoring of multiple metabolites. The approach has been shown to provide good selectivity/sensitivity for metabolites discovery and quantitation. However, LC has limitations for rapid and high-throughput analysis due to the time-consuming nature of chromatography. A comprehensive review [25, 26] of LC capabilities shows that total gradient times for a typical analysis are on the order of 45–60 min for HPLC, scaling to 5–6 min for UHPLC/UPLC (UPLC™ Waters). This typical analytical cycle time for LC depends on the sample pretreatment, and is extended if the sample contains lipids, or if column holdover requires blank runs. The Waters UPLC analyses for qualitative profiling of the urine samples studied here required a 12 min cycle time; they were analyzed after protein precipitation and contained some lipids. Furthermore, the samples are so complex that rapidly eluting compounds are affected by charge competition at the 0.5 mL/min flow rate and. The use of nanoLC to reduce ion suppression was not considered because run duration would have been doubled or tripled [3, 27]. In large commercial operation, several time-sliced LC systems can be multiplexed to a single MS system, but in typical facilities, these factors would hinder the application of LC-MS in clinical and field assessment of radiation damage where the rapid screening of a large number of subjects is of the essence.

DMS-MS technology is capable of increasing the speed, simplicity, and scalability of screening diagnostics [28], thereby improving the rate at which a determination of the need for medical countermeasures to radiation exposure can be made. Unlike LC which is a liquid phase separation technique, the separations in DMS take place in the gas phase during the transit through the ion filter (5–10 msec) and are based on the ions' properties such as structure, polarizability and cluster affinity. DMS therefore offers orthogonal separation and serves as a prefilter to MS [29, 30]. The total analysis time for a DMS-MS analysis is determined by the required averaging time for each compound (6 s in this application) times the number of compounds, and by the time to switch the flow and ion signal between samples. The reduction in chemical background from DMS improves the signal to noise

(S/N) ratio, and prevents contamination of the MS [31]. Isobaric and isomeric compounds often can be separated with DMS, even when structural differences are minor [32–34]. Our laboratory has pioneered the use of DMS-MS as a high-throughput alternative to LC-MS for quantitative analysis in a variety of areas, including forensics [33, 34] and biomarkers associated with DNA damage from carcinogens [35, 36].

In an initial study with a smaller scope, we used SPE-DMS-MS to analyze two metabolic biomarkers in NHP urine, comparing control urines to samples obtained after 10 Gy irradiation. Because that study was limited to only two doses and only examined two exposure levels (control and the high 10 Gy level), the results could not have medical relevance. That paper demonstrated that an efficient and effective SCX-SPE extraction for sample cleanup allows the combined SCX-SPE and DMS-MS methods to provide a rapid and accurate method of detection and quantitation of hypoxanthine and trimethyllysine in urine matrices [28].

The effectiveness of SPE for sample concentration and reduction of matrix effects has been demonstrated in studies by Mallet et al. [37], and by Dams et al. [38], among others. SPE has been compared to cleanup by protein precipitation, dilution, and direct flow injection. SPE is seen to have a more effective and better targeted result than protein precipitation. Mallet et al. also note that ion exchange, as in our strong cation exchange SCX-SPE, is particularly effective at reducing matrix effects. Ion suppression in ESI by matrix effects is described in Panuwet et al. and occurring in three stages: (1) charged matrix molecules prevent analyte molecules from reaching charges on the droplet surface, (2) competition within the droplet for charge, and (3) competition for charge in the gas phase. These matrix effects are all reduced at lower flow rates because of smaller droplet size and greater available charge per molecule, as discussed by K. Tang et al. [39]. Our current study and the previous one both use a reduced flow rate of 300 nL/min; typical LC flow rates are about 1000X higher. When the concentration of the analytes is high, as in the case of urinary creatinine, simple dilution in solvent is sufficient to completely eliminate matrix effects (see Stahnke et al. [40], as well as Dams et al. [38]), because it increases the available droplet charge per analyte molecule and prevents analyte-matrix interactions.

As an additional motivation for the current work, the protocol used in the previous work was designed for single marker analysis since different gas-phase modifiers were used for the two compounds (isopropanol and ethyl acetate), preventing simultaneous analysis, while the lack of intermediate radiation dose levels limited practical utility. The relationship between the biomarkers of radiation is complicated by dependence on the radiation type, dose rate, delay in acquiring samples, and other factors, and hence any variation in the concentration of a single marker, observed with restricted dose coverage, cannot be directly attributed to radiation damage. For example, compounds such as citrulline [41–45] and taurine [12, 46–48] are common markers of radiation exposure across several species, but their levels may also be perturbed by diet or other conditions [21, 49, 50]. Therefore, an approach based on the simultaneous monitoring of multiple radiation markers would ensure a more reliable and accurate assessment of radiation effects. In line with this goal, we present here a re-designed DMS-MS method for the simultaneous monitoring of several known markers in NHP urine. If the SPE cleanup method is effective for the metabolites of interest, there is no intrinsic

limit to the number of compounds that can be dealt with in this way, especially since the time required increases only incrementally with the number of analytes.

Experimental

Urine Samples and Sample Relevance

Urine samples were obtained after the completion of a recent study by Pannkuk et al. [3] of the long-term radiation response in non-human primates (NHP) (rhesus macaque animals (*Macaca mulatta*) of Chinese origin. The current method development did not involve animal care or the use of radiation. Full information on animal and radiation protocols is given in the Pannkuk et al. [3] reference. This study differed from earlier work in that it focuses on the relatively long term 7-day response with urine sample collection at 7 d only, while earlier studies addressed responses up to 72 h post-exposure. The whole-body doses administered from a ^{60}Co gamma source at 0.5 Gy/min (0 Gy, 2 Gy, 4 Gy, 6 Gy, 7 Gy, and 10 Gy) cover a range that is NHP-appropriate [51–54], and ^{60}Co radiation approximates the spectrum of weapons detonation [53]. The LD50/60 level (50% mortality at 60 d) is at approximately 7 Gy, and the change from 10% to 90% mortality occurs over a range of less than 2 Gy [53]. Mortality rate also has a dependence on the level of supportive treatment provided [51]. Similar human response occurs at doses which are lower by a factor of 1.5–2.0 [55, 56].

Chemicals and SPE Cartridges

Formic acid, acetonitrile (ACN), water (all LC-MS grade), and ammonium hydroxide were purchased from Fisher Scientific (Atlanta, GA, USA); hypoxanthine- $^{15}\text{N}_4$, xanthine- $^{15}\text{N}_2$ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); creatinine, creatine, hypoxanthine, trimethyllysine (N6,N6,N6-trimethyl-L-lysine), isobutyrylcarnitine, xanthine, xanthurenic acid were purchased from Sigma-Aldrich (Milwaukee, WI, USA); creatinine- $^2\text{H}_3$, creatine- $^2\text{H}_3$, isobutyrylcarnitine- $^2\text{H}_6$, xanthurenic acid- $^2\text{H}_4$ and trimethyllysine- $^2\text{H}_9$ were purchased from Santa Cruz Biotechnology; SCX SPE cartridges (1.5 mL size) were manufactured by Grace Davison Discovery Sciences (Deerfield, IL, USA) and purchased from Fisher Scientific (Atlanta, GA, USA).

DMS-MS Instrumentation

The DMS-MS instrumentation has been previously described [28]. Briefly, a prototype DMS API 3000 triple quadrupole mass spectrometer (AB SCIEX, Concord, ON, Canada) with DMS integrated into the Analyst 1.5.2 software was used for separation and detection. The separation voltage (SV) of the DMS has a range of 0 to 5000 V and –100 to +100 V for the compensation voltages (COV) applied across a gap of 1 mm, DMS filter length 15 mm, filter width 10 mm. The specific voltages for both SV and COV were determined for individual compounds. SV was applied as a two-harmonic waveform difference between 6 and 3 MHz waveforms [57], with the magnitude given as the signed difference between the minimum and maximum voltage applied across the 1 mm gap between the two planar DMS metal plates. Therefore, a setting of 4000 V corresponds to a high voltage of +2667 V/mm followed by a low voltage of –1333 V/mm (when COV = 0 V), as illustrated in Kafle et al. [58]. Electrospray ionization (ESI) with a voltage of ± 3500 V (± 2500 V difference between emitter and curtain plate on MS) was used for both positive and negative modes. The

stainless-steel emitter, 30 μm internal diameter, 50 mm length was purchased from Thermo Electron North America LLC (West Palm Beach, FL, USA) and used for the generation of electrospray. A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used for direct infusion at 300 nL/min. The SCIEX micro-spray emitter holder provided N_2 nebulizing at 1000 cc/min. The N_2 curtain gas flow was 1000 cc/min and heated to 85 $^\circ\text{C}$ with a homemade heated metal tube, so that both the 600 cc/min (vacuum drag) DMS transport gas and the 400 cc/min curtain gas counter-flow for ion desolvation were both maintained at that temperature. At a DMS transport gas temperature of 85 $^\circ\text{C}$, the E/N (E is the strength of electric field, N is the drift/transport gas number density) value in Townsends of the 4000 V setting corresponds to a peak value of 130 Td, and the 2000 V setting to a peak value of 65 Td. Measurements were made on the intensity of the parent ion MS peak, not in MS/MS mode, because the separation efficiency of the SPE preparation and the DMS ion selectivity were found to be sufficient for the analysis. In comparing SID-SPE-DMS-MS to SID-LC-MS/MS, the SPE step may be compared to LC in that sample complexity is reduced, and DMS to the Q_1 quadrupole filtration step in that ions are selected and chemical noise reduced, then both methods use a final MS detection. This demonstrates that the SID-SPE-DMS-MS could be applied in a field-portable device without MS/MS capability in the future.

Urine Samples

NHP urine samples were obtained from Georgetown University. The tested samples were a subset from a nonhuman primate (NHP) study of long-term radiation response [3]. Animal work conformed to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, and experimental protocols were reviewed and approved by an Institutional Animal Care and Use Committee. Further details on collection and handling of these samples are given in that reference [3] and succeeding work [18, 19].

The samples were from male rhesus monkeys and were all acquired 7-days post-exposure then stored at $-80\text{ }^\circ\text{C}$.: control samples from three sham-exposed subjects, and samples from subjects who were exposed to 2, 4, 6, and 7 Gy (3 subjects each dose), and 10 Gy (2 subjects). The 10 Gy sample had been analyzed previously by our group [25] for hypoxanthine only. Exposed subjects received 2–10 Gy exposures from a ^{60}Co gamma source; the control group received the same handling, but no irradiation.

Sample Preparation for Calibration Curves

Creatinine—The creatinine calibration sample was prepared separately from the six other calibration samples. Initially, a series of neat creatinine standard solutions in 50% ACN/ water + 0.1% formic acid ranging in concentration from 0.25 mM to 64 mM were prepared. Each solution was first diluted 1250-fold with 50% ACN+0.1% formic acid. Subsequently, 100 μL of the 1250-fold diluted solution and 100 μL internal standard (IS) solution (creatinine- $^2\text{H}_3$ in 50% ACN+0.1% formic acid) were combined, resulting in a 2500-fold dilution. When used with the NHP urine samples, this level of dilution is known to be sufficient to eliminate matrix suppression or enhancement [38, 40, 59]. The final solution was then centrifuged at 10,000 rcf (g) for 3 min to remove any particles before MS analysis.

Other markers—A single series of neat standard solutions of the normal isotopes (in water adjusted to pH 2 with formic acid) containing all six markers were prepared in which the concentrations of creatine, hypoxanthine, trimethyllysine, isobutyrylcarnitine and xanthine ranged up to 1000 μ M; and xanthurenic acid up to 500 μ M. A single reference IS solution used for both calibration and quantitation was prepared (in pH 2 water) containing isotopically labeled analogs of all compounds. Subsequently, 40 μ L blank human urine + 40 μ L standard solution from each concentration + 40 μ L IS solution + 80 μ L pH 2 water were combined. The above combined 200 μ L solution was applied to each preconditioned (with 1 mL pH 2 water two times) Grace SCX-SPE cartridge, washed with 1 mL pH 9 water (adjusted with ammonium hydroxide) two times, then eluted with 1 mL 30% ammonium hydroxide two times. All eluted samples were dried down with Speed-Vac at 50 °C and reconstituted with a final volume of 500 μ L 50% acetonitrile + 0.1% FA. All reconstituted samples were then centrifuged at 10,000 rcf (g) for 3 min to remove any particles before DMS-MS analysis.

Sample Preparation for Analysis of NHP Control and Irradiated Urine Samples

Creatinine—NHP urine samples for creatinine analysis were prepared separately from the other seven markers in the same way as for the calibration curves. First, 5 μ L NHP urine sample + 995 μ L solvent (50% ACN+0.1% formic acid) were combined. 16 μ L of the above solution was then combined with 84 μ L solvent (50% ACN+0.1% formic acid), then the latter 100 μ L solution was combined with 100 μ L creatinine- $^2\text{H}_3$ (1.6 μ M) solution, resulting in effectively 2500-fold dilution in total. The final solutions were then centrifuged at 10,000 rcf (g) for 3 min to remove any particles before MS analysis.

Other seven markers—40 μ L NHP urine + 40 μ L IS solution + 120 μ L pH 2 water were combined. Concentrations in the IS solution are described in the previous section. The above combined 200 μ L solutions were applied to each preconditioned (with 1 mL pH 2 water two times) Grace SCX-SPE cartridge, washed with 1 mL pH 9 water (adjusted with ammonium hydroxide) two times, then eluted with 1 mL 30% ammonium hydroxide two times. All eluted samples were dried down with speed-vac under 50 °C and reconstituted with a final volume of 500 μ L 50% acetonitrile + 0.1% FA. All reconstituted samples were then centrifuged at 10,000 rcf (g) for 3 min to remove any particles before DMS-MS analysis.

Data Acquisition and Processing

All data were acquired by Sciex Analyst (version 1.5.2) and processed by Excel. A queue consisting of seven DMS-MS methods (each compound has a unique DMS-MS method, seven compounds have seven different DMS-MS methods) was built with the following order: xanthine, xanthurenic acid, creatine, hypoxanthine, trimethyllysine, kynurenic acid, isobutyrylcarnitine. The signal for kynurenic acid was too low for analysis, due to low levels in the samples. Each DMS-MS method used 6 seconds for signal acquisition. The signal intensity of each marker and its isotope labeled internal standard were recorded. The intensity ratio of analyte/internal standard was calculated as Y value; the concentration of spiked analytes was used as X value to generate calibration curves. For NHP urine samples, the detected intensity ratio of analyte/internal standard was then applied into the calibration curve as the Y value and the corresponding X value (concentration) was calculated.

Results and Discussion

The structures of the biomarkers quantified in this study are shown in Scheme 1. As we discuss in detail below, creatinine is quantified separately, and plays a dual biological role, traditionally a measure of the current kidney glomerular filtration rate (GFR), and also an indication of kidney damage in the case of high radiation dose.

DMS-MS is a two-dimensional separation technique that depends on the DMS parameters compensation voltage (COV) at an optimized separation voltage (SV)), and the m/z of the ion. This COV value represents the field dependence of the ion mobility coefficient, and is determined by the ion's physical and electric properties such as polarity, size, shape, dipole moment, etc., which are largely independent of the ion's m/z ratio [60]. The combination of the COV value and m/z of each biomarker defines the conditions for separation of the components in a mixture. Thus, before proceeding with the analysis of urine samples from NHP exposed to radiation, it was necessary to optimize the DMS-MS parameters for each biomarker in the seven compounds mixture bearing in mind the need for separation and detection.

DMS parameters which select for a particular analyte are properties of individual gas-phase ions and the bulk transport gas. The DMS conditions have no dependence on concentrations in the electrospray solution or the liquid matrix since ESI ion desolvation is aided by our use of microflow (300 nL/min) and by the counter-flow of curtain gas at the curtain plate in the Sciex DMS API 3000. However, intensities are subject to competitive ionization in electrospray, so a biological matrix should be used. Accordingly, a blank human urine sample spiked with 150 μM of each of the seven biomarkers in human urine was used for this purpose. The sample was purified by SCX-SPE as described in the experimental section and analyzed by DMS-MS. The optimal DMS-MS parameters such as m/z , COV and full width at half maximum (FWHM) of COV for each biomarker are summarized in Table 1 and were used to construct the 2D separation picture shown in Figure 1. While DMS is not the equal of chromatography, DMS performs a similar filtration process and can resolve closely related compounds in mixtures as shown in [61, 62] for the separation of substances of forensic interest.

Because of its very high concentration in human and NHP urine, creatinine could be quantified by direct dilution, since high dilution reduces matrix effects effectively [63–66]. The creatinine calibration curve was generated using a 2500-fold dilution in solvent (50% ACN + 0.1% formic acid) with IS addition.

Calibration Curves

Following determination of the DMS-MS parameters of all biomarkers, calibration curves covering a ca. 100-fold dynamic range were constructed. Because sufficient NHP control urine was not available and since matrix effect comparisons supporting this substitution will be available in another paper from this group [67], human urine solutions containing various concentrations of each analyte in the range of 10–1000 μM were prepared including isotopically labeled standards (IS). As shown in Figure 2, good linearity with R^2 values of 0.99 or better were obtained for most compounds. Each point in the calibration curve was

analyzed in triplicate and, in general, after completion of the SPE cleanup step, for any given sample, the DMS-MS analysis of all six markers in one sample was accomplished in less than 2 minutes. Moreover, the entire process (including sample preparation) of generation of the six calibration curves (kynurenic acid is not shown here) shown in Figure 2 was completed in less than two hours, providing a notable advantage over LC for the simultaneous quantitation of multiple targeted biomarkers in biological matrices. Creatinine was calibrated after 2500X solvent dilution as mentioned previously, because of its high concentration in urine. The calibration curve for kynurenic turned out to be unnecessary to present here because its concentration in the NHP urine samples was below the LOD of the calibration curve.

Analysis of Urine from NHP Exposed to Radiation

Following generation of the calibration curves, the 7-day post-exposure NHP urine samples were analyzed with addition of the same target-combined IS solution used to generate the calibration curves. The run is made by rapidly switching DMS and MS settings during infusion to the microspray ESI source. Switching time between compound settings is about 50 ms. The sequential nature of DMS filtration can put the method at a disadvantage to UPLC TOF in some cases, especially when many compounds are being analyzed.

The biomarker concentrations which were determined by the SID-SPE-DMS-MS calibration curves are shown in Figure 2. The same samples had been previously assessed in a global-profiling metabolomics study by Pannkuk et al. [3]. The DMS-MS results are presented in Figures 3, 4, and 5, showing marker concentrations as a function of exposure. Creatine, trimethyllysine, xanthine, and xanthurenic acid, show a distinct rise in the concentrations with increasing dose, especially in the transition from 6 Gy to 7 Gy. There is a higher level of biological variation in the sensitive 6–7 Gy range, followed by a lower level of variation at 10 Gy. The dose at which countermeasures are essential is in the range that approaches or exceeds the LD 30 level of approximately 6.5 Gy in NHP. As a result, these results are able, *prima facie*, to provide a direct and immediate identification of the patients most in need of treatment.

Creatinine Normalization in Metabolomics

Creatinine is the cyclic anhydride of creatine. Creatine and phosphocreatine are a major part of the ATP-ADP energy cycle in muscle tissue. Creatine, which is synthesized in the kidney, liver, and pancreas, is degraded, at a nearly constant rate of 1–2% per day, into creatinine, which is treated as a waste product, filtered by the glomerulus in the kidney and excreted in the urine. For a healthy individual, and depending on muscle mass, the amount of creatinine generated per day is constant. The serum concentration of creatinine is also held constant by the interplay of generation and excretion and by additional feedback mechanisms which have not been fully explored [68, 69].

Serum creatinine levels are one of the simplest measure of kidney function or glomerular filtration rate (GFR). Other measures of kidney function include 24 h urine creatinine, and the “creatinine clearance” value involving both serum and urine measurements. Agreement among the different approaches is less than perfect [70, 71], and the 24 h urine creatinine is

considered to be the best reference. The importance of creatinine in two roles as a measure of glomerular filtration rate and the observation of high levels as indicative of kidney damage, is documented in medical/clinical text books, both for humans [72–74] and for animals [75]. Another important effect of kidney damage is the presence of albumin or other proteins in the urine (proteinuria). This measurement of proteinuria is definitive but is somewhat lacking in sensitivity, so combinations of protein tests and urine creatinine are also used for assessing kidney function [75, 76]. No measurements of protein levels were made in these or the global profiling studies.

Normalization of urine metabolomics results by creatinine levels is useful to compensate for individual fluid intake levels, but would distort an important response if kidney damage has resulted from the very high 6–10 Gy radiation doses. This ambiguity is specific to urine samples. Normalization of serum metabolomics results by creatinine level is not generally necessary because of creatinine homeostasis (60–110 μ M male, 45–90 μ M female, (BMJ Best Practice, <http://bestpractice.bmj.com/topics/en-gb/935>, accessed Feb. 23, 2018). Urinary creatinine levels in a human population cover a much wider range than is found in serum because of influences that are not generally present in animal model populations that are cared for in a highly controlled environment, and are given a regulated diet. A high-volume toxicology lab familiar with human samples indicates “Normal random urine creatinine concentrations range from 40–300 mg/dL in males and 37–250 mg/dL in females” (Redwood Toxicology Lab., https://www.redwoodtoxicology.com/docs/resources/creatinine_interpretation.pdf, accessed Feb. 23, 2018), a factor of 10, rather than a factor of 2 (serum). Fluid intake can cause levels of creatinine and other metabolites to vary by factors of two or more in a single day (for example, see Mericq et al. [70]), and dietary supplements or medications can also have an effect. Urine samples from a human population vary widely in tonicity due to fluid intake patterns that are either episodic or habitual, and so can vary the absolute concentrations of targeted metabolites. Even a model animal population exposed to a significant stressor may change fluid intake patterns, and thus glomerular filtration rate (GFR), during the experiment due to the exposure.

Our current study examines the NHP urinary response to radiation, but is intended to lead to applications in radiation biodosimetry for humans that make use of human urine samples. The NHP subject population was acclimated to a uniform and controlled environment, so one might expect that creatinine normalization would not make a major difference in the trends and diagnostic information seen in the data. Waikar et al. found that normalization to creatinine can somewhat improve the performance of biomarker indicators [77]. On the other hand, omitting creatinine normalization allows creatinine to be used independently as an indicator of radiation-induced kidney damage at the higher exposure levels either alone or through the creatine-creatinine dependency. The independent proteinuria test was not a part of the current protocol.

In summary, normalization to urinary creatinine might be useful in improving the performance of urinary metabolic biomarkers, but creatinine itself can be an indication of kidney damage or of a larger response leading to lower fluid intake. In the interests of providing a more complete picture, and because of the dual role of creatinine, we are

providing the results both without and with creatinine normalization. The comparison of the two treatments do not show a particular benefit from creatinine normalization.

Trends in Biomarker Radiation Responses

Figure 3 displays the total level quantitation in μM for the six simultaneously analyzed radiation biomarkers. Creatinine, which was measured separately, is shown in Figure 4. Results above 1000 μM are extrapolations that must be regarded as approximate. For isobutyrylcarnitine, the low values at high radiation level are also an extrapolation. The endogenous level is treated by adding the calibration intercept, based on the discussion in the paper by Mani, Abbatiello and Carr [78]. Since the calibration curve is based on spike level added to a matrix, using the calibration curve gives the level relative to the level in the matrix. Total concentration is the sum of calibration curve value and the calibration intercept. This correction is not significant for upregulated compounds, but, is significant in the case of isobutyrylcarnitine, where the total concentration decreases to near zero at 10 Gy. Figure 5 shows the same data normalized to creatinine. In Figure 5, 10 Gy is not shown because creatinine of those samples was not measured due to depletion of the 10 Gy samples. These figures show the average concentrations over all the NHP which received the particular dose, with vertical bars for biological variation shown as the standard error of the mean (SEM). The technical variation in these measurements, determined from three repetitions of each measurement is always below 10%, and generally 5% or less. The data used to generate the plots are shown in Table 2.

The dose response of the biomarkers in the same NHP urine samples of the same cohort obtained by DMS-MS was compared to that obtained in the related study conducted by LC-MS [3]. The observed trends for four of the markers: creatinine, xanthurenic acid, creatine, and hypoxanthine were strikingly similar while the data for xanthine were somewhat inconclusive. It should be emphasized however that the DMS-MS analysis provided absolute quantitative information based on the stable isotope dilution (SID) method using isotopically labeled internal standards while the LC-MS comparisons were based on the detection of uncalibrated responses.

Differences between SPE-DMS-MS and LC-MS results

DMS-MS and LC-MS approaches have previously been compared for accuracy and sensitivity in a paper on the analysis of dG-ABP DNA adducts in calf-thymus DNA by Kafle et al. [79] They showed that the DMS-MS/MS results on a Agilent cube-chip ion trap agreed with those from a well-known previously-validated LC-MS/MS method developed and used on a much more sensitive system, and that the use of DMS filtration to eliminate background ions was essential to quantitation by DMS-MS/MS in the ion trap. DMS is especially useful on ion-trap systems because it reduces the trapped load of unwanted matrix ions [32].

In the current work, two compounds appear to disagree with the trends seen in NHP in the global metabolomics profiling by Pannkuk et al.[3]: trimethyllysine (TML), and isobutyrylcarnitine (iBCAR). TML was identified as radiation-responsive in human samples by Laiakis et al. [22] but was not reported in the same NHP samples that we analyzed, and

for which a clear dose response was observed (Figure 3) under the highly-selective DMS conditions of SV = 4000V (Table 1). Possible explanations for this include the following.

1. Spectral congestion and competitive ionization in the profiling analysis may be important. The reversed-phase (RP) LC conditions used in the profiling runs did not retain TML or the smaller carnitines, so TML appears with the very first ions at 0.26 m in [22]. A review of the raw data from the Pannkuk et al. profiling runs shows that TML can be identified tentatively from exact mass and isotope ratio in the first eluted ions where it appears as a very minor component in a congested spectrum of other polar compounds competing for charge. TML may not have been reliably identified or detected by software under these conditions. Alternative conditions have been reported that provide good retention and separation of TML and several carnitines [80], and compare GC and LC-based methods [81].
2. Protein binding may also contribute. TML plays an important role in epigenetics, and is bound strongly by $(\text{CH}_3)_3\text{N}^+$ cation-aromatic π interactions to a number of proteins [82, 83]. The LC-MS protocol, which uses a protein precipitation, might remove some TML, while the current work uses a SCX-SPE preparation which was selected for its ability to concentrate the targeted metabolites.

Our quantitative demonstration of a TML radiation response is consistent with its role as a carnitine precursor since acyl carnitines are also known to be markers of radiation exposure as shown by quantitative measurements in our recent paper which also discusses the matrix effect on calibration [67], and with its observation as a marker in human TBI patients [22].

The declining level seen for isobutyrylcarnitine in Figure 3 also appears to differ from the rising level seen for the sum of the MS/MS-indistinguishable butyrylcarnitine and isobutyrylcarnitine in the profiling study. With the assistance of B. B. Schneider of Sciex, we have found that the two butyrylcarnitine isomers are approximately 75/25 separated under our high-field DMS conditions, which were optimized for the iBCAR isomer. The possibility of a difference between the responses of the two butyrylcarnitines should be investigated in a future study.

Comparing throughput of SPE-DMS-MS and LC-MS

The strong similarity of the biodosimetry data obtained independently by two different laboratories each using a different method (LC-MS and SPE-DMS-MS) warrants a more quantitative assessment of the benefits that may be realized from the high-throughput potential of DMS-MS. The advantages of DMS-MS in rapid analysis are summarized and compared with LC-MS in Table 3, which shows in detail the times involved in every key step in the analysis by the respective methods. Both the development of all calibration curves for the seven markers and the analysis of the NHP urine samples by DMS-MS was completed in less than 30 minutes (not including SPE), which is about 7.5-fold faster than UPLC and 30-fold faster than nanoLC. Radiation biodosimetry requires fast and high-throughput screening, a requirement well fulfilled with DMS-MS. The SPE approach is a little more time-consuming than protein precipitation, however, it provides significantly

higher extraction and clean-up efficiency. In addition, the speed of SPE can be improved by using SPE automation devices such as Agilent's Rapid-Fire.

Biological significance

Development of high-throughput methodology for identification of markers that display a dose dependent change that is unique to ionizing radiation exposure rather than disease or trauma is of major biological significance, especially when viewed in the context of a potential nuclear accident. The compounds selected for this study and their biological pathways have already been identified in both NHP and human TBI [3, 22]. They are indicative of purine catabolism [22, 84] and perturbation of fatty acid β -oxidation pathway [3, 22], metabolic alterations commonly implicated in IR exposure.

Trimethyllysine (TML) is a lysine derivative that is coenzyme for fatty acid oxidation, and the precursor for L-carnitine biosynthesis [22], occurring primarily in the liver and kidneys in that role. Carnitine is produced after TML is converted to 3-Hydroxy-N^ε-trimethyllysine (EC 1.14.11.8), to 4-N-trimethylaminobutyraldehyde, and finally γ -butyrobetaine (EC 1.2.1.47). Carnitine's important biological role is the transportation of long chain acyl groups from fatty acids (as acylcarnitines) in the mitochondria to be catabolized to acetyl-CoA that can then be utilized in the tricarboxylic acid (TCA) cycle for energy metabolism. In addition, TML is an important epigenetic modifier, which as a histone modifier is part of chromatin regulation. Through that connection, the increase of TML at very high radiation levels could also involve chromosomal or DNA damage.

Beside the direct action of IR on DNA to induce DNA lesions (single and double strand breaks), an indirect consequence of IR exposure is the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) from water hydrolysis, which result in damage to DNA, proteins, and lipids. With regards to DNA integrity and repair, the purine hypoxanthine is either formed from deaminated adenine (EC 3.5.4.2) or from inosine (EC 3.2.2.8, EC 3.2.2.1, and EC 3.2.2.2) and is a marker of DNA damage from oxidative stress [3, 22]. Hypoxanthine is a mutagenic product in that it may pair with cytosine leading to downstream errors in DNA transcription. In addition to IR exposure, hypoxanthine has been proposed as a biomarker in myriad diseases including hepatocellular carcinoma [85], kidney diseases [86], Parkinson's disease [87] and childhood pneumonia [88], among others.

Estimating the radiation dose

The quantitative analysis of samples obtained by the SCX-ESI-DMS-MS method can be used to test simple approaches to the determination of doses and dose thresholds from the measurements. Obtaining a dose estimate from the measurements requires both changes in mean concentrations, and limited biological variation to the same exposure. Because the change in mortality rate is very steep in the 6–7 Gy exposure range, samples obtained at the 7-day point may have greater variability because the population is beginning to resolve into groups by outcome. Earlier time points might be more uniform in response.

Figure 6 shows a scale-independent overview of the relationship among the concentrations of all 7 markers as a function of dose, as determined by Pearson correlation of each sample with the average control (0 Gy) levels. Each correlation calculation uses two lists or vectors.

First, the list of mean concentration of all markers for a particular (NHP id, dose), and a similar list for the mean of all control NHP subjects. The correlation calculated by the Pearson method is the vector dot product of the control list with the list of a (NHP id, dose) subject, normalized by the product of the vector lengths. Creatinine was included, using an estimated value for 10 Gy. The MATLAB function *corr* was used for the computation. This metric has a useful sensitivity to the 2 Gy level separately from the other doses. This is a useful observation that identifies the lowest but significant exposure. There are unusual responses in the 6 Gy and 7 Gy groups that highlight the increased response variability to doses in that transitional exposure range. The 6–7 Gy marks the onset of reduced survivability, the transition from LD10 to LD90, with individual variation in outcome.

In order to use the observed data to assign a dose level or a dose relative to a threshold to each sample, a simple linear-discriminant-analysis Gaussian mixture model (LDA-GMM) has been tested (Matlab, MathWorks, Natick, MA, function *ClassificationDiscriminant.fit*). Models were generated for three different goals: A. complete dose identification, B. identify doses greater than 3 Gy, and C. identify doses greater than 5 Gy, with the results shown in Table 4. The expected error rate can be estimated from the Gaussian mixture parameters based on the overlap between the distributions, providing an expected accuracy on additional data. Even though there are only 2 errors in the training set, the expected error rate is high for full classification (71%), but it is 18% for the threshold goals, a level which would be useful for medical triage. The data used in training the LDA-GMM is shown below.

This exercise in dose estimation is only schematic because results from other samples in the study are not yet available to use as a test set. The additional samples must be quantified and used for further model development. However, the reasonable error estimate of the threshold models is encouraging. It is also important to consider data from other studies which include the early time response from 6 h to 72 h post-exposure to understand the development of the response, leading to an outcome. Both the average response development and the breadth of individual variability are important in radiation dose estimation and medical treatment.

Conclusion

We have conducted an evaluation of the potential use of DMS-MS for simultaneous quantitation of multiple analytes in urine. The results presented in this study demonstrate that the entire screening process can be significantly expedited and that our DMS-MS procedures are already well positioned to deal effectively with particular multiplexed assays.

In this method, micro-flow infusion is analyzed for the target and isotopic standard signals sequentially. DMS parameters are the same for normal and isotopic signals, measured at the two *m/z* values. The controlling Sciex Analyst software allows DMS parameters and MS *m/z* value to be changed rapidly during the run, taking 50 ms between the six seconds we devoted to averaging each particular target. Because DMS ion filtration selects one target at a time, the sequential nature of DMS-MS analysis can put the approach at a disadvantage to an optimized UPLC method followed by Q-TOF for analysis of many compounds. In addition, sample pretreatment is an important part of the DMS method because competitive ionization occurs during ESI of the infused sample. In chromatography, the ideal case would

allow well-separated compounds to be ionized with good efficiency. However, the data acquired for global profiling after a simple protein precipitation [3] shows 50 or more strong peaks at many retention times. The effective peak capacity of DMS is lower than LC, but the use of SPE pretreatment and microspray ionization can compensate for that limitation. DMS modifiers increase peak capacity and reduce chemical noise but can also reduce signal by charge competition, which varies with the target and the modifier. Some compounds in the target list were sensitive to modifier concentration which cannot be changed rapidly, so no modifier was used.

In this application, six biomarkers associated with radiation damage were measured in under two minutes, excluding the SPE time which can be automated. The trends observed were in general agreement with independently conducted LC-MS studies. Isobutyrylcarnitine is an exception which may be due to DMS resolution of structural isomers not resolved by MS. In summary, we have incorporated radiation biomarkers in a single panel and provided this more rapid and high-throughput DMS-MS method that can be translated from the laboratory setting to field-deployable systems.

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References

1. International Atomic Energy Agency. Incident and Emergency Preparedness and Response - 2016. 2017. <https://www.iaea.org/sites/default/files/publications/reports/2016/gc61-3-incident-and-emergency.pdf>
2. Singh VK, Newman VL, Romaine PL, Hauer-Jensen M, Pollard HB. Use of biomarkers for assessing radiation injury and efficacy of countermeasures. *Expert Rev Mol Diagn.* 2016; 16:65–81. [PubMed: 26568096]
3. Pannkuk EL, Laiakis EC, Authier S, Wong K, Fornace AJ Jr. Global Metabolomic Identification of Long-Term Dose-Dependent Urinary Biomarkers in Nonhuman Primates Exposed to Ionizing Radiation. *Radiat Res.* 2015; 184:121–133. [PubMed: 26230079]
4. Pannkuk EL, Fornace AJ Jr, Laiakis EC. Metabolomic applications in radiation biodosimetry: exploring radiation effects through small molecules. *Int J Radiat Biol.* 2017:1–26.
5. Goudarzi M, Mak TD, Chen C, Smilenov LB, Brenner DJ, Fornace AJ. The effect of low dose rate on metabolomic response to radiation in mice. *Radiation and environmental biophysics.* 2014; 53:645–657. [PubMed: 25047638]
6. Laiakis EC, Pannkuk EL, Diaz-Rubio ME, Wang YW, Mak TD, Simbulan-Rosenthal CM, Brenner DJ, Fornace AJ Jr. Implications of genotypic differences in the generation of a urinary metabolomics radiation signature. *Mutat Res.* 2016; 788:41–49. [PubMed: 27040378]
7. Laiakis EC, Strassburg K, Bogumil R, Lai S, Vreeken RJ, Hankemeier T, Langridge J, Plumb RS, Fornace AJ Jr, Astarita G. Metabolic phenotyping reveals a lipid mediator response to ionizing radiation. *J Proteome Res.* 2014; 13:4143–4154. [PubMed: 25126707]
8. Laiakis EC, Wang YW, Young EF, Harken AD, Xu Y, Smilenov L, Garty GY, Brenner DJ, Fornace AJ Jr. Metabolic Dysregulation after Neutron Exposures Expected from an Improvised Nuclear Device. *Radiation research.* 2017
9. Leung KS, Fong BM. LC-MS/MS in the routine clinical laboratory: has its time come? *Analytical and bioanalytical chemistry.* 2014; 406:2289–2301. [PubMed: 24337187]

10. Mak TD, Tyburski JB, Krausz KW, Kalinich JF, Gonzalez FJ, Fornace AJ Jr. Exposure to ionizing radiation reveals global dose- and time-dependent changes in the urinary metabolome of rat. *Metabolomics*. 2015; 11:1082–1094. [PubMed: 26557048]
11. Strathmann FG, Hoofnagle AN. Current and future applications of mass spectrometry to the clinical laboratory. *American journal of clinical pathology*. 2011; 136:609–616. [PubMed: 21917684]
12. Tyburski JB, Patterson AD, Krausz KW, Slavik J, Fornace AJ Jr, Gonzalez FJ, Idle JR. Radiation metabolomics. 1. Identification of minimally invasive urine biomarkers for gamma-radiation exposure in mice. *Radiat Res*. 2008; 170:1–14. [PubMed: 18582157]
13. Laiakis EC, Hyduke DR, Fornace AJ. Comparison of mouse urinary metabolic profiles after exposure to the inflammatory stressors gamma radiation and lipopolysaccharide. *Radiat Res*. 2012; 177:187–199. [PubMed: 22128784]
14. Goudarzi M, Weber WM, Mak TD, Chung J, Doyle-Eisele M, Melo DR, Strawn SJ, Brenner DJ, Guilmette RA, Fornace AJ Jr. A Comprehensive Metabolomic Investigation in Urine of Mice Exposed to Strontium-90. *Radiat Res*. 2015; 183:665–674. [PubMed: 26010713]
15. Goudarzi M, Weber WM, Mak TD, Chung J, Doyle-Eisele M, Melo DR, Brenner DJ, Guilmette RA, Fornace AJ Jr. Metabolomic and lipidomic analysis of serum from mice exposed to an internal emitter, cesium-137, using a shotgun LC-MS(E) approach. *J Proteome Res*. 2015; 14:374–384. [PubMed: 25333951]
16. Goudarzi M, Weber WM, Chung J, Doyle-Eisele M, Melo DR, Mak TD, Strawn SJ, Brenner DJ, Guilmette R, Fornace AJ Jr. Serum Dyslipidemia Is Induced by Internal Exposure to Strontium-90 in Mice, Lipidomic Profiling Using a Data-Independent Liquid Chromatography-Mass Spectrometry Approach. *J Proteome Res*. 2015; 14:4039–4049. [PubMed: 26262552]
17. Goudarzi M, Weber W, Mak TD, Chung J, Doyle-Eisele M, Melo D, Brenner DJ, Guilmette RA, Fornace AJ. Development of urinary biomarkers for internal exposure by cesium-137 using a metabolomics approach in mice. *Radiation research*. 2014; 181:54–64. [PubMed: 24377719]
18. Pannkuk EL, Laiakis EC, Mak TD, Astarita G, Authier S, Wong K, Fornace AJ. A lipidomic and metabolomic serum signature from nonhuman primates exposed to ionizing radiation. *Metabolomics*. 2016; 12:1–11.
19. Pannkuk EL, Laiakis EC, Authier S, Wong K, Fornace AJ Jr. Targeted Metabolomics of Nonhuman Primate Serum after Exposure to Ionizing Radiation: Potential Tools for High-throughput Biodosimetry. *RSC Adv*. 2016; 6:51192–51202. [PubMed: 28367319]
20. Pannkuk EL, Laiakis EC, Authier S, Wong K, Fornace AJ Jr. Gas Chromatography/Mass Spectrometry Metabolomics of Urine and Serum from Nonhuman Primates Exposed to Ionizing Radiation: Impacts on the Tricarboxylic Acid Cycle and Protein Metabolism. *J Proteome Res*. 2017; 16:2091–2100. [PubMed: 28351153]
21. Johnson CH, Patterson AD, Krausz KW, Kalinich JF, Tyburski JB, Kang DW, Luecke H, Gonzalez FJ, Blakely WF, Idle JR. Radiation metabolomics. 5. Identification of urinary biomarkers of ionizing radiation exposure in nonhuman primates by mass spectrometry-based metabolomics. *Radiation research*. 2012; 178:328–340. [PubMed: 22954391]
22. Laiakis EC, Mak TD, Anizan S, Amundson SA, Barker CA, Wolden SL, Brenner DJ, Fornace AJ Jr. Development of a metabolomic radiation signature in urine from patients undergoing total body irradiation. *Radiat Res*. 2014; 181:350–361. [PubMed: 24673254]
23. Ding S, Schoenmakers I, Jones K, Koulman A, Prentice A, Volmer DA. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. *Anal Bioanal Chem*. 2010; 398:779–789. [PubMed: 20628873]
24. French D. Advances in Clinical Mass Spectrometry. *Adv Clin Chem*. 2017; 79:153–198. [PubMed: 28212711]
25. Guillardie D, Ruta J, Rudaz S, Veuthey JL. New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches. *Anal Bioanal Chem*. 2010; 397:1069–1082. [PubMed: 19998028]
26. Guillardie D, Veuthey JL. Chapter 1: The Theory and Practice of UHPLC and UHPLC-MS. In: Byrdwell WC, Holcapek M, editors *Extreme chromatography: faster, hotter, smaller*. AOCS Press; Urbana, IL: 2012. v485

27. Randall KL, Argoti D, Paonessa JD, Ding Y, Oaks Z, Zhang Y, Vouros P. An improved liquid chromatography–tandem mass spectrometry method for the quantification of 4-aminobiphenyl DNA adducts in urinary bladder cells and tissues. *Journal of Chromatography A*. 2010; 1217:4135–4143. <http://dx.doi.org/10.1016/j.chroma.2009.11.006> [PubMed: 19932483]
28. Chen Z, Coy SL, Pannkuk EL, Laiakis EC, Hall AB, Fornace AJ, Vouros P. Rapid and High-Throughput Detection and Quantitation of Radiation Biomarkers in Human and Nonhuman Primates by Differential Mobility Spectrometry-Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*. 2016:1–11. [PubMed: 27126468]
29. Krylov E, Nazarov E. Electric field dependence of the ion mobility. *International journal of mass spectrometry*. 2009; 285:149–156.
30. Schneider BB, Covey TR, Coy SL, Krylov EV, Nazarov EG. Chemical effects in the separation process of a differential mobility/mass spectrometer system. *Analytical chemistry*. 2010; 82:1867–1880. [PubMed: 20121077]
31. Schneider B, Covey T, Coy S, Krylov E, Nazarov E. Planar differential mobility spectrometer as a pre-filter for atmospheric pressure ionization mass spectrometry. *Int J Mass spectrom*. 2010; 298:45–54. [PubMed: 21278836]
32. Hall AB, Coy SL, Kafle A, Glick J, Nazarov E, Vouros P. Extending the dynamic range of the ion trap by differential mobility filtration. *J Am Soc Mass Spectrom*. 2013; 24:1428–1436. [PubMed: 23797861]
33. Hall AB, Coy SL, Nazarov EG, Vouros P. Rapid separation and characterization of cocaine and cocaine cutting agents by differential mobility spectrometry-mass spectrometry. *Journal of forensic sciences*. 2012; 57:750–756. [PubMed: 22235847]
34. Hall AB, Coy SL, Nazarov E, Vouros P. Development of rapid methodologies for the isolation and quantitation of drug metabolites by differential mobility spectrometry - mass spectrometry. *International journal for ion mobility spectrometry: official publication of the International Society for Ion Mobility Spectrometry*. 2012; 15
35. Klaene JJ, Sharma VK, Glick J, Vouros P. The analysis of DNA adducts: the transition from (32)P-postlabeling to mass spectrometry. *Cancer letters*. 2013; 334:10–19. [PubMed: 22960573]
36. Klaene JJ, Flarakos C, Glick J, Barret JT, Zarbl H, Vouros P. Tracking matrix effects in the analysis of DNA adducts of polycyclic aromatic hydrocarbons. *J Chromatogr A*. 2016; 1439:112–123. [PubMed: 26607319]
37. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Commun Mass Spectrom*. 2004; 18:49–58. [PubMed: 14689559]
38. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid. *J Am Soc Mass Spectrom*. 2003; 14:1290–1294. [PubMed: 14597119]
39. Tang K, Page JS, Smith RD. Charge competition and the linear dynamic range of detection in electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom*. 2004; 15:1416–1423. [PubMed: 15465354]
40. Stahnke H, Kittlaus S, Kempe G, Alder L. Reduction of matrix effects in liquid chromatography-electrospray ionization-mass spectrometry by dilution of the sample extracts: how much dilution is needed? *Anal Chem*. 2012; 84:1474–1482. [PubMed: 22243135]
41. Waterfield C, Delaney J, Kerai M, Timbrell J. Correlations between in vivo and in vitro effects of toxic compounds: Studies with hydrazine. *Toxicol In Vitro*. 1997; 11:217–227. [PubMed: 20654308]
42. Jones JW, Tudor G, Bennett A, Farese AM, Moroni M, Booth C, MacVittie TJ, Kane MA. Development and validation of a LC-MS/MS assay for quantitation of plasma citrulline for application to animal models of the acute radiation syndrome across multiple species. *Anal Bioanal Chem*. 2014; 406:4663–4675. [PubMed: 24842404]
43. Jones J, Scott A, Tudor G, Xu PT, Jackson I, Vujaskovic Z, Booth C, MacVittie T, Ernst R, Kane M. Identification and Quantitation of Biomarkers for Radiation-induced Injury via Mass Spectrometry. *Health Phys*. 2014; 106:106–119. [PubMed: 24276554]

44. Goudarzi M, Chauthé S, Strawn SJ, Weber WM, Brenner DJ, Fornace AJ. Quantitative Metabolomic Analysis of Urinary Citrulline and Calcitroic Acid in Mice after Exposure to Various Types of Ionizing Radiation. *Int J Mol Sci*. 2016; 17:17.
45. Bujold K, Hauer-Jensen M, Donini O, Ramage A, Hartman D, Hendrickson HP, Stamatopoulos J, Naraghi H, Pouliot M, Ascah A, Sebastian M, Pugsley MK, Wong K, Authier S. Citrulline as a Biomarker for Gastrointestinal-Acute Radiation Syndrome: Species Differences and Experimental Condition Effects. *Radiat Res*. 2016; 186:71–78. [PubMed: 27351760]
46. Doheny M, Waterfield C, Timbrell J. The effects of the beta 2-agonist drug clenbuterol on taurine levels in heart and other tissues in the rat. *Amino Acids*. 1998; 15:13–25. [PubMed: 9871484]
47. Timbrell JA, Waterfield CJ, Draper RP. Use of urinary taurine and creatine as biomarkers of organ dysfunction and metabolic perturbations. *Comp Haematol Int*. 1995; 5:112–119.
48. Waterfield C, Asker D, Timbrell J. Does urinary taurine reflect changes in protein metabolism? A study with cycloheximide in rats. *Biomarkers*. 1996; 1:107–114. [PubMed: 23888921]
49. Jones JW, Tudor G, Bennett A, Farese AM, Moroni M, Booth C, MacVittie TJ, Kane MA. Development and validation of a LC-MS/MS assay for quantitation of plasma citrulline for application to animal models of the acute radiation syndrome across multiple species. *Analytical and bioanalytical chemistry*. 2014; 406:4663–4675. [PubMed: 24842404]
50. Piton G, Manzon C, Cypriani B, Carbonnel F, Capellier G. Acute intestinal failure in critically ill patients: is plasma citrulline the right marker? *Intensive care medicine*. 2011; 37:911–917. [PubMed: 21400011]
51. Farese A, Brown C, Smith C, Gibbs A, Katz B, Johnson C, Prado K, MacVittie T. The Ability of Filgrastim to Mitigate Mortality Following LD50/60 Total-body Irradiation Is Administration Time-Dependent. *Health Phys*. 2014; 106:39–47. [PubMed: 24276548]
52. Farese AM, Cohen MV, Katz BP, Smith CP, Jackson W 3rd, Cohen DM, MacVittie TJ. A nonhuman primate model of the hematopoietic acute radiation syndrome plus medical management. *Health Phys*. 2012; 103:367–382. [PubMed: 22929469]
53. MacVittie T, Farese A, Jackson W. The Hematopoietic Syndrome of the Acute Radiation Syndrome in Rhesus Macaques: A Systematic Review of the Lethal Dose Response Relationship. *Health Phys*. 2015; 109:342–366. [PubMed: 26425897]
54. Thrall K, Love R, O'Donnell K, Farese A, Manning R, MacVittie T. An Interlaboratory Validation of the Radiation Dose Response Relationship (DRR) for H-ARS in the Rhesus Macaque. *Health Phys*. 2015; 109:502–510. [PubMed: 26425909]
55. Graessle D, Dorr H, Bennett A, Shapiro A, Farese A, MacVittie T, Meineke V. Comparing the Hematopoietic Syndrome Time Course in the NHP Animal Model to Radiation Accident Cases From the Database Search. *Health Phys*. 2015; 109:493–501. [PubMed: 26425908]
56. Dorr H, Lamkowski A, Graessle D, Bennett A, Shapiro A, Farese A, Garofalo M, MacVittie T, Meineke V. Linking the Human Response to Unplanned Radiation and Treatment to the Nonhuman Primate Response to Controlled Radiation and Treatment. *Health Phys*. 2014; 106:129–134. [PubMed: 24276556]
57. Krylov E, Coy S, Vandermeij J, Schneider B, Covey T, Nazarov E. Selection and generation of waveforms for differential mobility spectrometry. *Rev Sci Instrum*. 2010; 81:024101. [PubMed: 20192506]
58. Kafle A, Coy SL, Wong BM, Fornace AJ Jr, Glick JJ, Vouros P. Understanding gas phase modifier interactions in rapid analysis by differential mobility-tandem mass spectrometry. *J Am Soc Mass Spectrom*. 2014; 25:1098–1113. [PubMed: 24452298]
59. Ferrer C, Lozano A, Agüera A, Giron AJ, Fernandez-Alba AR. Overcoming matrix effects using the dilution approach in multiresidue methods for fruits and vegetables. *J Chromatogr A*. 2011; 1218:7634–7639. [PubMed: 21820661]
60. Shvartsburg AA, Tang K, Smith RD. Two-Dimensional Ion Mobility Analyses of Proteins and Peptides. In: Lipton M, Paša-Tolić L, editors *Mass Spectrometry of Proteins and Peptides*. Humana Press, part of Springer Science; New York: 2009. 417–445.
61. Hall AB, Coy SL, Nazarov EG, Vouros P. Rapid Separation and Characterization of Cocaine and Cocaine Cutting Agents by Differential Mobility Spectrometry–Mass Spectrometry. *J Forensic Sci*. 2012; 57:750–756. [PubMed: 22235847]

62. Hall AB, Coy SL, Nazarov EG, Vouros P. Development of rapid methodologies for the isolation and quantitation of drug metabolites by differential mobility spectrometry – mass spectrometry. *Int J Ion Mobil Spec.* 2012; 15:151–156.
63. Huskova R, Chrastina P, Adam T, Schneiderka P. Determination of creatinine in urine by tandem mass spectrometry. *Clinica chimica acta; international journal of clinical chemistry.* 2004; 350:99–106. [PubMed: 15530465]
64. Fraselle S, De Cremer K, Coucke W, Glorieux G, Vanmassenhove J, Schepers E, Neirynck N, Van Overmeire I, Van Loco J, Van Biesen W, Vanholder R. Development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry method to measure creatinine in human urine. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences.* 2015; 988:88–97. [PubMed: 25756209]
65. Kwon W, Kim JY, Suh S, In MK. Simultaneous determination of creatinine and uric acid in urine by liquid chromatography-tandem mass spectrometry with polarity switching electrospray ionization. *Forensic Sci Int.* 2012; 221:57–64. [PubMed: 22503624]
66. Klaene JJ, Flarakos C, Glick J, Barret JT, Zarbl H, Vouros P. Tracking Matrix Effects in the Analysis of DNA Adducts of Polycyclic Aromatic Hydrocarbons. *Journal of chromatography A.* 2016; 1439:112–123. [PubMed: 26607319]
67. Vera NB, Pannkuk EL, Laiakis EC, Fornace AJ Jr, Erion DM, Coy SL, Pfefferkorn JA, Vouros P. Differential Mobility Spectrometry (DMS) Reveals the Elevation of Urinary Acetylcarnitine in Non-Human Primates (NHPs) Exposed to Radiation. *J Mass Spectrom.* 2018 in press.
68. Lamb EJ, Price CP. *Tietz Fundamentals of Clinical Chemistry.* 6th. Saunders/Elsevier; St. Louis Missouri: 2007. Creatinine, Urea, and Uric Acid; 363–372.
69. Burtis C, Bruns D. *Tietz Fundamentals of Clinical Chemistry.* 6th. Saunders/Elsevier; St. Louis, Missouri: 2007.
70. Mericq MV, Cutler GB Jr. High fluid intake increases urine free cortisol excretion in normal subjects. *J Clin Endocrinol Metab.* 1998; 83:682–684. [PubMed: 9467592]
71. Hafeez AR, Idrees MK, Akhtar SF. Accuracy of GFR estimation formula in determination of glomerular filtration rate in kidney donors: Comparison with 24 h urine creatinine clearance. *Saudi J Kidney Dis Transpl.* 2016; 27:320–325. [PubMed: 26997385]
72. Feher J. *Quantitative Human Physiology.* Second. Academic Press; Boston: 2017. 7.4 - Tubular Reabsorption and Secretion; 719–729.
73. Feher JJ. *Quantitative Human Physiology: An Introduction.* Elsevier Academic Press; 2012. 7.3 - Glomerular Filtration; 633–641. [ScienceDirect.com](https://www.sciencedirect.com)
74. Dasgupta A, Wahed A. *Clinical Chemistry, Immunology and Laboratory Quality Control.* Elsevier; 2014. Renal Function Tests; 197–212.
75. Kain R, Pagitz M. Chronic Kidney Failure Affects Humans and Other Mammals. In: Jensen-Jarolim E, editor *Comp Med.* Springer; 2017.
76. Wang MK, White C, Akbari A, Brown P, Hussain N, Hiremath S, Knoll G. Utilizing Estimated Creatinine Excretion to Improve the Performance of Spot Urine Samples for the Determination of Proteinuria in Kidney Transplant Recipients. *PLoS One.* 2016; 11:11.
77. Waikar SS, Sabbiseti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. *Kidney Int.* 2010; 78:486–494. [PubMed: 20555318]
78. Mani DR, Abbatiello SE, Carr SA. Statistical characterization of multiple-reaction monitoring mass spectrometry (MRM-MS) assays for quantitative proteomics. *BMC Bioinformatics.* 2012; 13(Suppl 16):S9.
79. Kafle A, Klaene J, Hall AB, Glick J, Coy SL, Vouros P. A differential mobility spectrometry/mass spectrometry platform for the rapid detection and quantitation of DNA adduct dG-ABP. *Rapid Commun Mass Spectrom.* 2013; 27:1473–1480. [PubMed: 23722681]
80. Hirche F, Fischer M, Keller J, Eder K. Determination of carnitine, its short chain acyl esters and metabolic precursors trimethyllysine and gamma-butyrobetaine by quasi-solid phase extraction and MS/MS detection. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877:2158–2162.
81. Lu Y, Li N, Gao L, Xu YJ, Huang C, Yu K, Ling Q, Cheng Q, Chen S, Zhu M, Fang J, Chen M, Ong CN. Acetylcarnitine Is a Candidate Diagnostic and Prognostic Biomarker of Hepatocellular Carcinoma. *Cancer Res.* 2016; 76:2912–2920. [PubMed: 26976432]

82. Hughes RM, Benshoff ML, Waters ML. Effects of chain length and N-methylation on a cation- π interaction in a beta-hairpin peptide. *Chemistry (Easton)*. 2007; 13:5753–5764.
83. Zheng X, Wu C, Ponder JW, Marshall GR. Molecular dynamics of beta-hairpin models of epigenetic recognition motifs. *J Am Chem Soc*. 2012; 134:15970–15978. [PubMed: 22934656]
84. Tyburski JB, Patterson AD, Krausz KW, Slavik J, Fornace AJ Jr, Gonzalez FJ, Idle JR. Radiation metabolomics. 2. Dose- and time-dependent urinary excretion of deaminated purines and pyrimidines after sublethal gamma-radiation exposure in mice. *Radiat Res*. 2009; 172:42–57. [PubMed: 19580506]
85. Wu H, Xue R, Dong L, Liu T, Deng C, Zeng H, Shen X. Metabolomic profiling of human urine in hepatocellular carcinoma patients using gas chromatography/mass spectrometry. *Anal Chim Acta*. 2009; 648:98–104. [PubMed: 19616694]
86. Tanaka K, Tani Y, Asai J, Nemoto F, Kusano Y, Suzuki H, Hayashi Y, Asahi K, Katoh T, Miyata T. Skin autofluorescence is associated with renal function and cardiovascular diseases in pre-dialysis chronic kidney disease patients. *Nephrology Dialysis Transplantation*. 2011; 26:214–220.
87. Johansen KK, Wang L, Aasly JO, White LR, Matson WR, Henchcliffe C, Beal MF, Bogdanov M. Metabolomic profiling in LRRK2-related Parkinson's disease. *PLoS One*. 2009; 4:e7551. [PubMed: 19847307]
88. Laiakis EC, Morris GA, Fornace AJ, Howie SR. Metabolomic analysis in severe childhood pneumonia in the Gambia, West Africa: findings from a pilot study. *PLoS One*. 2010; 5

Figure 1.
2D separation of biomarkers by DMS-MS. Compensation voltage (COV) positions and widths were obtained from experimental data on the pure compounds.

Figure 2.

Calibration curves. The slope standard errors 1 to 3% of the slope value (Excel, LINEST).

The error bars on the triplicate measurements are mostly too small to be drawn visibly.

Creatinine was calibrated by SID-MS after 2500 X dilution, others by SID-SPE-DMS-MS in human urine. The calibrated dynamic range for creatinine is 0.25–64 mM. The other calibrated dynamic ranges are 31.25–1000 μ M (creatine), 15.625–500 μ M (xanthurenic acid), and 15.625–1000 μ M for others. For the six compounds, the IS solution used for calibration and quantitation contained all markers.

Figure 3. Metabolite radiation response in NHP urine samples quantified by SID-SPE-DMS-MS as μM concentrations. Error bars show the standard error of the mean (SEM). See text for discussion.

Figure 4. NHP urine creatinine concentration (mM) as a function of radiation dose, with biological variation as SEM. The 10 Gy measurement was not made because of depletion of the very limited 10 Gy urines in other measurements.

Figure 5.

Dose response in NHP urine samples quantified by DMS-MS, normalized to creatinine (μM/mM creatinine). No data are shown for 10 Gy due to the lack of 10 Gy creatinine (Figure 4). Error bars (SEM) show the biological variation. Technical variation of repeated measurements is typically below 5%.

Figure 6. Pearson correlation of the metabolite levels of each sample with the mean of control samples.

Scheme 1.
Structures of radiation biomarkers.

DMS-MS parameters for 8 radiation biomarkers (Positive mode: $[M+H]^+$; Negative mode: $[M-H]^-$).**Table 1**

Compound	ESI Polarity	<i>m/z</i>	Internal Standard	<i>m/z</i>	SV (V)	COV (V)	FWHM
Creatinine	Positive	114	Creatinine- ² H ₃	117	0	0	N/A
Creatine	Positive	132	Creatine- ² H ₃	135	2000	-1	1
Hypoxanthine	Positive	137	Hypoxanthine- ¹⁵ N ₄	141	2000	0.4	1.5
Trimethyllysine	Positive	189	Trimethyllysine- ² H ₉	198	4000	5	1
Isobutyrylcarnitine	Positive	232	Isobutyrylcarnitine- ² H ₆	238	4000	4	1
Xanthine	Negative	151	Xanthine- ¹⁵ N ₂	153	2000	-0.4	3
Xanthurenic acid	Negative	204	Xanthurenic acid- ² H ₄	208	2000	0.3	2.5

Table 2

Biologically averaged concentrations of the radiation biomarkers. The 6 Gy to 7 Gy range corresponds to the LD30 to LD50 exposure for the rhesus macaque NHP species. The biological variation (SEM) is higher at 6 or 7 Gy than at either lower or higher exposures for creatine, TML, xanthine, and xanthurenic acid, as shown in Figure 3. (TML is N6,N6,N6-trimethyl-L-lysine, iBCAR is isobutyryl/carnitine).

Biological average concentration									
Dose(Gy)	Samples	μM					mM		
		creatine	hypoxanthine	TML	iBCAR	xanthine	xanthurenic acid	creatinine	
0	3	434.2	132.6	98.8	31.9	176.6	45.6	27.1	
2	3	144.7	152.6	50.8	34.7	223.7	54.8	15.9	
4	3	312.2	85.0	99.3	39.3	192.6	59.3	19.6	
6	3	992.1	118.9	36.9	31.5	163.7	78.9	11.3	
7	3	4623.1	134.7	1543.6	13.9	520.2	100.8	20.0	
10	2	8155.0	141.5	1193.2	0.8	470.2	233.8	–	
Standard error of the mean (SEM)									
Dose(Gy)	Samples	μM					mM		
		creatine	hypoxanthine	TML	iBCAR	xanthine	xanthurenic acid	creatinine	
0	3	131.43	18.63	11.94	10.07	36.91	8.01	8.14	
2	3	22.54	38.39	15.96	2.18	15.89	5.95	1.10	
4	3	127.73	29.22	55.97	13.70	43.67	9.16	3.99	
6	3	733.97	43.58	9.77	8.52	48.19	6.66	1.29	
7	3	1424.59	22.60	779.95	9.68	167.00	76.48	6.80	
10	2	817.85	42.95	124.21	1.82	59.22	46.06	–	

Table 3

Comparison of LC-MS and DMS-MS shows a factor of 7.5–30 increase in throughput using SPE-DMS-MS, with SCX-SPE assumed to be performed in batch mode. UPLC and nanoLC timings include time for two blank runs. DMS-MS timing is for our prototype system and includes 10 s to generate a stable ESI signal; 6 s data acquisition per marker, 42 s in total; 60 s for cleaning the sample transfer line. The sample preparation timing in this table is based on a batch of approximately 20 samples of the type used in this study.

Analysis platform	LC-MS	DMS-MS
Sample preparation	Protein precipitation. 15–25 min for all samples	Solid phase extraction (SPE) 1–2 hrs for all samples
Sample volume for single analysis	100 µL	40 µL
Detection on mass spec/sample	UPLC: 15–30 min [3] NanoLC: 60 min [27]	~2 min
Daily throughput on mass spec/day (24 hrs)	UPLC: 48–96 samples NanoLC: 24 samples	~720 samples

Table 4

Linear-discriminant Gaussian-mixture models using the observed concentrations (μM) for three different goals: A. exact dose, B. dose exceeds 3 Gy, and C. dose exceeds 5 Gy. The expected error rate for additional observations is derived from the Gaussian mixture parameters. The expected error rates for threshold detection goals would be useful for rapid pre-clinical screening. See Table 5 for the measurements by dose and subject.

Goal	Control	Error count				Model quality	
		2 Gy	4 Gy	6 Gy	7 Gy	10 Gy	Est. error rate
A. Exact dose?	0	1	0	1	0	0	0.71
B. dose exceeds 3 Gy?	0	1	0	0	0	0	0.18
C. dose exceeds 5 Gy?	0	0	0	1	0	0	0.18

Table 5

Biological variation for marker concentrations (μM) in the NHP 7-day urine samples. The technical variation is generally on the order of 5% or less. Misclassified samples in Table 4 are “2007” and “4006”. Limited 10 Gy sample was exhausted before creatinine measurements, so values are note given.

Concentration	by NHP	Name	creatinine	hypoxanthine	TML	isobutyrylcarnitine	xanthine	xanthurenic acid	creatinine
-----μM-----									
Control		C1	420.3	113.7	121.3	43.2	224.5	47.5	43.4
		C2	668.4	114.3	80.6	11.8	201.3	58.5	19.5
		C3	213.8	169.9	94.5	40.8	104.0	30.9	18.6
2 Gy		2005	159.4	201.0	48.9	37.7	247.8	46.6	14.7
		2006	100.5	180.0	24.1	36.0	193.7	51.4	14.9
		2007	174.3	76.8	79.2	30.5	229.7	66.3	18.1
4 Gy		3005	208.7	137.3	41.6	66.4	136.3	41.3	20.0
		3006	161.6	81.5	45.0	29.4	162.8	70.9	12.5
		3008	566.2	36.3	211.2	22.2	278.6	65.9	26.3
6 Gy		4004	2455.2	45.6	54.3	28.2	97.4	81.9	11.6
		4005	364.0	114.8	20.5	18.7	136.4	66.2	8.9
		4006	157.2	196.4	36.1	47.7	257.5	88.7	13.3
7 Gy		5006	4651.7	155.9	189.0	33.1	346.5	89.2	15.3
		5007	4594.6	158.7	2890.8	6.6	693.9	329.3	33.4
		5008	349.6	89.5	1551.1	2.0	119.6	112.3	11.3
10 Gy		6007	8972.9	98.6	1317.4	2.6	410.9	187.8	–
		6008	7337.1	184.5	1069.0	–1.0	529.4	279.9	–