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Pilot Metabolome-Wide Association Study of Benzo(a)pyrene in Serum from Military Personnel

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Abstract

Objective—A pilot study was conducted to test the feasibility of using Department of Defense Serum Repository (DoDSR) samples to study health and exposure-related effects.

Methods—Thirty unidentified human serum samples were obtained from the DoDSR and analyzed for normal serum metabolites with high-resolution mass spectrometry and serum levels of free benzo(a)pyrene (BaP) by gas chromatography-mass spectrometry. Metabolic associations with BaP were determined using a metabolome wide association study (MWAS) and metabolic pathway enrichment.

Results—The serum analysis detected normal ranges of glucose, selected amino acids, fatty acids, and creatinine. Free BaP was detected in a broad concentration range. MWAS of BaP showed associations with lipids, fatty acids, and sulfur amino acid metabolic pathways.

Conclusion—The results show the DoDSR samples are of sufficient quality for chemical profiling of DoD personnel.

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Keywords

Department of Defense Serum Repository; High-resolution Metabolomics; Polycyclic Aromatic Hydrocarbons; Exposure Monitoring; Bioeffect; Exposome; Deployment

Introduction

The Department of Defense Serum Repository (DoDSR) contains serum samples from service personnel originally collected as part of mandatory HIV testing and later extended to include pre- and post-deployment samples (1). While not currently utilized for routine biomonitoring, serum samples stored in the repository have potential as a valuable resource for assessing deployment-related exposures, which has been recognized as an important need in the National Academy of Sciences 2000 report “Strategies to Protect the Health of Deployed U.S. Forces: Detecting, Characterizing, and Documenting Exposures” (2). The recent availability of high-throughput chemical profiling platforms with expanded coverage, such as high-resolution metabolomics (HRM), now makes possible cost-effective environmental chemical surveillance and bioeffect monitoring using DoDSR samples (3, 4). HRM, which utilizes liquid chromatography and high-resolution mass spectrometry, provides quantitative measure of a large number (10,000-15,000) of endogenous and exogenous chemicals in biological samples that can be used to profile biomarkers of exposure, effect, and alterations in metabolism consistent with disease (3-5).

Before the DoDSR can be appropriately used as a resource for evaluating exposure, there is a need to test sample suitability for chemical profiling. Since HRM is a new technology, there is no way to assess sample alterations occurring during storage; however, quality can still be evaluated by testing for expected metabolic patterns and biological associations with targeted measurement of specific exposure biomarkers. Thus, combining the metabolic measurements obtained from HRM and levels of exposure biomarkers measured using either HRM or other platforms, it is possible to test for exposure-related metabolic changes. Comparison of *in vivo* metabolic associations measured in the serum can then be interpreted in light of known toxicological targets and toxicodynamics, allowing assessment of sample suitability for measuring biologically relevant effects.

Deployment-related exposures have the potential to occur from a number of sources. Of particular interest is identifying respiratory exposure to combustion products generated from burn pits, which is suspected as a possible risk factor for new onset asthma and respiratory symptoms (6, 7). Serum levels of BaP, a carcinogenic polycyclic aromatic hydrocarbon arising from the combustion of organic material, was selected as an exposure biomarker to test the suitability of DoDSR samples for elucidating biological response to environmental exposures. Previous biomonitoring studies have shown that BaP was measureable in serum because of respiratory exposure to smoke, exhaust and incomplete combustion (8-11). During the course of deployment, service personnel are known to potentially experience a wide range of BaP exposures from different sources (12, 13), although due to the unknown time of collection for serum samples in this study it is not possible to link measured BaP to exposures during deployment.

The present pilot study was designed to evaluate the feasibility of using DoDSR samples for measuring biomarkers of exposure and associated biological response. HRM chemical profiling of thirty unidentified DoDSR samples was completed to evaluate 1) whether metabolites could be reliably detected in the serum samples 2) serum levels of parent BaP and 3) metabolic pathway alterations associated with serum BaP detected using a metabolome-wide association study (MWAS) framework (14-16). It is important to recognize that although serum levels of BaP represent exposure biomarkers relevant to deployed troops, it is not possible to directly associate serum BaP levels with deployment due to the unidentified nature of the samples. By testing the suitability of DoDSR for metabolic studies, we hope to define sample quality for metabolic studies and use as a resource for chemical profiling.

Materials and Methods

Serum Samples

Thirty unidentified serum samples (i.e., information on source, date of collection and meta-data is unknown) were obtained from the DoDSR for BaP targeted quantification and HRM. The repository consists of approximately 50 million serum samples originally collected for mandatory HIV testing in armed forces personnel (17). Following collection, samples were stored at -30°C. Prior to analysis, specimens were thawed at 4°C, 500 µL was aliquoted into separate microfuge tubes after vortexing, refrozen and shipped on dry ice.

BaP targeted quantification

BaP was measured using sample preparation and GC-MS methodologies optimized for analysis of DoDSR samples (18) and based upon previously established methods. (19). Detailed method information can be found in the supporting materials. Following addition of an internal standard solution consisting of deuterated anthracene (d₁₀), serum polycyclic aromatic hydrocarbons (PAHs) were first extracted using Triton X-100, followed by hexane. Sample extracts were analyzed by a Thermo Fisher Trace gas chromatograph interfaced to a quadrupole mass spectrometer (Thermo Fisher DSQ) operated in selective ion monitoring mode (SIM). Concentration of BaP was determined by comparing the peak intensities to a five-point calibration curve. Method fitness of purpose and detection limit was evaluated by spiking serum samples with BaP at two concentration ranges, with BaP recovery exceeding >80%. Method limit of detection was 0.0028 ng/mL.

High-resolution metabolomics

Samples were prepared for metabolomics analysis using established methods described elsewhere (20-22); detailed method information can be found in the supporting materials. Briefly, serum aliquots were removed from storage at -80°C, and thawed on ice, upon which 65 µL of serum was added to 130 µL of acetonitrile containing a mixture of stable isotopic standards, vortexed and allowed to equilibrate for 30 min. Following protein precipitation, triplicate 10 µL aliquots were analyzed by reverse-phase C₁₈ liquid chromatography with Fourier transform mass spectrometry (Dionex Ultimate 3000, Q-Exactive, Thermo) operated in positive electrospray ionization mode and resolution of 70,000 (FWHM) over mass-to-charge (m/z) range of 85-1,250. Samples were analyzed in batches of 15, in addition to a

quality control (QC) pooled reference sample included at the beginning and end of the analytical batch for quantification and standardization. Raw data files were extracted using hybrid apLCMS (23) with modifications by xMSanalyzer (24), with each unique ion defined by mass-to-charge ration (m/z), retention time and ion abundance.

Tentative chemical identifications for all detected features was completed by matching the accurate mass m/z features to commonly observed adducts at a ± 5 ppm mass error threshold using the *feat.batch.annotation.KEGG()* function in xMSanalyzer (24) and the Kyoto Encyclopedia for Genes and Genomes (KEGG) database. Since no information was known about the origin of the serum samples, 15 common human endogenous metabolites were quantified within the 30 samples by reference standardization (25). Serum concentrations from the National Health and Nutrition Examination Survey (NHANES) and Human Metabolome Database (HMDB) were included for comparison.

Feature selection

Sparse partial least squares (sPLS) regression analysis (26) was utilized to identify features associated with BaP serum levels. The sPLS regression analysis performs a supervised dimensionality reduction approach to maximize covariance between the continuous response variable (BaP serum level) and predictor variables (m/z feature intensity profile) (27). The top 100 features were selected as a manageable number for subsequent analyses. Feature selection was completed using the *sPLS()* function within the R package mixOmics (28). Visualization of the sPLS significant features and grouping based on similarity in expression were evaluated with unsupervised hierarchical clustering. The corresponding heat map was plotted using dendrogram clustering and z-score normalized intensity profiles. BaP levels were then categorized based on sample clustering.

Metabolite correlation and pathway enrichment

To evaluate pathway correlations of metabolites associated with measured BaP levels, a metabolome-wide Spearman correlation analysis was applied to the 100 m/z features selected with sPLS to capture associations within the raw data. Spearman rank correlation coefficient and Benjamini and Hochberg (29) false discovery rate were calculated pair-wise for the sPLS selected features using the R package MetabNet (30). Correlations with $|r| \geq 0.6$ and FDR $\leq 20\%$ were selected to test for metabolic pathway enrichment in Mummichog (31). Enriched pathways were selected at significance score ≤ 0.05 . A select number of metabolite matches were confirmed by MS/MS, and compared to reference standards (when available) or database spectra (32-34). Confirmed identifications by MS/MS included the metabolites selected for absolute quantification, in addition to linoleate, sphinganine, sphingosine, cholesterol, acetyl-carnitine, threonine, dihydrobiopterin, histamine, 4-chlorophenylacetate and methionine.

Results

Targeted quantification of endogenous human metabolites

Table 1 shows the average concentration of 15 metabolites selected for quantification by reference standardization, which includes essential and non-essential amino acids, glucose,

fatty acids and metabolites known to be involved in various disease processes. Creatinine and creatine were detected at average concentrations of 93 ± 13 and $16 \pm 8 \mu\text{M}$, respectively. Due to the tightly regulated range of glucose in healthy individuals, serum glucose levels represent an excellent indicator of overall health. The average glucose level in these serum samples of $4312 \pm 822 \mu\text{M}$, which corresponds to a glucose level of 77 mg/dL and is within the range typically observed during a fasting glucose test (70-99 mg/dL). One sample had an extremely low glucose level of 4 mg/dL.

BaP targeted profiling

BaP was detected over a wide concentration range in the serum (Figure 1), with a minimum, 25th percentile, median, 75th percentile and maximum concentration (ng/mL) of 0.13, 1.45, 2.09, 3.18 and 37.2, respectively, corresponding to an average serum BaP concentration of $3.39 \text{ ng/mL} \pm 1.19 \text{ SE}$. The presence of outlier BaP levels was evaluated by subtracting or adding $2.0 \times \text{IQR}$ (interquartile range) from the 1st and 3rd BaP quartiles. For the thirty individuals, one serum level corresponding to 37.2 ng/mL exceeded the IQR limits. While biologically plausible, this sample was removed prior to sPLS analysis to avoid biasing results due to the study size.

High-resolution metabolomics

Following data extraction and alignment, 7,810 unique m/z features were detected with a median replicate relative standard deviation (RSD) of 19.6%. Technical replicates were averaged and filtered based on the requirement that each feature must be present in greater than 10% of the samples, resulting in 7,584 remaining features. Median relative standard deviation for triplicate measurements following filtering was 19.8%, providing a median standard error of the mean value of 11.4%. Annotation using m/z values included 6,010 unique KEGG IDs, which represented 70 metabolic pathways with ten or greater matches to metabolic intermediates present. These included 625 within the overall human metabolic map (Figure 2). It should be noted that these categories are estimates based upon accurate mass matches because confirmation of structures was not practical for this number of chemicals with the amount of sample available.

Serum BaP MWAS

To test for associations with BaP, a sPLS based MWAS was completed using as BaP serum levels as the continuous dependent variable and detected features as predictor variables. We selected the top 100 features from sPLS for further analysis, representing a manageable number for subsequent characterization. Average intensity of the selected features ranged over 500-fold, with 81% of the features corresponding to compounds with $m/z > 600$. Retention time distribution was skewed towards early eluting compounds, with 62% detected in 3 minutes or less. To determine if groups of metabolites are associated with BaP serum levels, we performed two-way unsupervised hierarchical clustering (Figure 3). The top 100 features grouped into 15 clusters (Supplementary Table S1), which represented both increased and decreased expression with BaP serum levels (left axis). Clustering of samples based upon sPLS selected metabolic profiles (horizontal axis) resulted in 4 separate groups based upon BaP levels, including a high-exposure group ($n=2$, average BaP serum concentration 5.77 ng/mL), mid-high exposure ($n=9$, average BaP serum concentration 3.06

ng/mL), mid-low exposure (n= 4, average BaP serum concentration 1.87 ng/mL) and low exposure (n=14, average BaP serum concentration 1.28 ng/mL). Clusters were used to define low (1.28 ng/mL; n= 11) and high (high + mid-high; average= 3.55 ng/mL; n= 14) BaP groups for comparing relative expression of metabolites in significant metabolic pathways.

To provide identification of sPLS features based upon m/z , the KEGG database was searched for matches to common adducts at a mass error threshold of 5 ppm (listed in Supplementary Table S1). Feature grouping by BRITE categorization (34), included lipids (n=34, 21.0%), phytochemicals (n=22, 13.6%), pesticides (n=7, 4.3%), compounds with biological roles (n=5, 3.1%), carcinogens (n=5, 3.1%), pharmaceuticals (n=5, 3.1%) and endocrine disrupting compounds (n=1, 0.6%), with the remaining 51.2% (n= 83) non-classified. The assigned identities of the annotated features include important biological metabolites and compounds originating from exogenous sources. Centrally acting, biological molecules important in overall metabolism and signaling include threonine (+H-H₂O), dihydrobiopterin (+H), histamine (+H), hexadecanol (+ACN+H), sphinganine (+H-H₂O), hydroxybutanoic acid (+H), linoleate (+H-H₂O), and 2-hydroperoxy-octadecatrienoic acid (+Na). Previous studies for this platform have confirmed identities for threonine, dihydrobiopterin, histamine, sphinganine and linoleate. Within the annotated features, exogenous chemicals were detected as well, including included chlordecone (+Na), cartap (+ACN+H), bendiocarb/dioxacarb (+ACN+Na), cymoxanil (+ACN+H), prometryn/terbutyn (+Na), imazethapyr (+ACN+Na), 4-chlorophenylacetate (+H-H₂O) and trimethoxytoluene (+H). Methoxypyrene (+ACN+H), a product of PAH metabolism, was also identified as one of the significant features. For the remaining 57 sPLS significant features, no matches were present in the KEGG database.

Metabolite correlation and pathway analysis

Metabolome-wide Spearman correlation analysis was applied to the m/z features selected with sPLS to test for correlations within the raw HRM data. An additional 388 m/z s were identified as associated with the sPLS features (Figure 4). The full list was utilized for pathway enrichment. One or more of the MWAS features were present in 58 of the 114 metabolic pathways. The top 15 identified pathways are shown in Figure 5. Seven were found to be enriched based on a significance score > 0.05 (filled bars); individual metabolites are listed in Supplementary Table S2. Enriched pathways included linoleate metabolism (p= 0.0017), carnitine shuttle (p= 0.0035), drug metabolism (p= 0.005), butanoate metabolism (p= 0.009), glycerophospholipid metabolism (p= 0.029), prostaglandin formation (p= 0.029) and methionine/cysteine metabolism (p= 0.043). Box and whisker plots of metabolites from these pathways are shown in Figure 6 and Figure 7.

Discussion

This pilot study analyzed 30 unidentified serum samples obtained from the DoDSR and measured free BaP, a PAH commonly found in diesel exhaust and linked to cancer in a number of organ systems, including liver and lung. BaP measured in serum was correlated with HRM profiles of metabolic pathway intermediates. MWAS of BaP showed associations with lipids, fatty acids, methionine and cysteine metabolic pathways and demonstrated

associations with metabolic pathways that have previously been connected with BaP exposure *in vivo* and *in vitro*.

Serum sample quality was assessed by comparing levels of representative human metabolites to published values for the general population. Quantified serum chemicals including glucose, creatinine, amino acids and carnitine were found in concentrations that are in the normal range for healthy individuals (Table 1) based on the average expected blood concentrations in HMDB (35) and the National Health and Examination Survey (NHANES). While the measured value of carnitine in this study was higher than the average value listed in HMDB, carnitine levels up to 79 μ M have been reported (36). The average glucose level was comparable to healthy individuals (70-99 mg/dL). One sample was observed to have a serum glucose level of 4 mg/dL, suggesting a sample collection artifact. When collecting blood serum, coagulant and storage conditions strongly influence inhibition of glycolysis. This has been observed to result in an 85% decrease in serum glucose levels following storage for 24 hours (37), and may be useful as a reliable marker of serum handling.

Biomonitoring of BaP exposure is most commonly accomplished by measuring hydroxylated urinary metabolites or formation of DNA adducts (38-40); however, serum concentrations of parent BaP can be utilized as a general indicator of respiratory and dietary exposures. BaP was one of the most commonly detected PAHs in post-mortem blood from male and female Caucasians and African Americans (41), which ranged in concentration from 0.6 ng/mL to 10.5 ng/mL. A similar concentration range of 0-3.7 ng/mL was reported by Al-Daghri et al. (11); however, Pleil et al. (8), reported median and maximum BaP levels of 0.019 and 0.195 ng/mL, respectively, in plasma obtained from a healthy, non-smoking, adult population with no recent history of remarkable exposures. Thus, reported measures of BaP vary widely, and further characterization of BaP distribution in general and exposed populations is required.

Detected features within the metabolic profile were observed to associate with BaP serum levels and included alterations in immune and neurotransmitter related pathways. Histamine, which acts as a localized mediator of inflammation through regulation of four receptors and the corresponding intracellular signals (42), was decreased with increasing BaP. Exposure to diesel fuel particles has been shown to increase allergic inflammation in both animal models and humans through increased local antigen-specific immunoglobulin E and cytokine release (43-45). In addition, animal and *in vitro* models of BaP exposure have indicated increases in histamine production following exposure periods (46, 47). While histamine was decreased in our study, association with BaP level nonetheless suggests changes in metabolic pathways important to immune response. Threonine, which is utilized for mucin synthesis and immunoglobulin A production, was increased with higher serum levels of BaP. Dihydrobiopterin, which is produced during synthesis of tyrosine, dopamine and serotonin, was also increased within the higher exposed group. Animal models indicate that elevated serum levels of dihydrobiopterin led to endothelial nitric oxide synthase dysfunction (48). Association of these metabolites with BaP suggests a possible relationship to both immune and cardiovascular function, consistent with known pathophysiology of respiratory exposure to combustion products.

Pathway enrichment identified associations with both lipid/fatty acid and oxidative metabolic pathway intermediates. For example, seven significant metabolic intermediates from glycerophospholipid metabolism (Figure 6) were identified in the enrichment analysis. Linoleate, a polyunsaturated, essential fatty acid utilized for biosynthesis of prostaglandins and cell membranes was decreased in the higher BaP groups, as well as sphinganine and sphingosine, which provide critical roles in ceramide lipid formation, and free cholesterol. Glycerophospholipid metabolism has previously been identified as an altered pathway following metabolic profiling of a BaP rat exposure model (49), and included differential expression of sphinganine and phytosphingosine. The authors attributed this to possible disruption to cell membranes based on the decreased levels of serum ceramide (d18:0/14:0) and increased sphinganine and phytosphingosine by sphingomyelinases alterations. This trend was not observed in the present study. Lipid profiling of human mammary cells dosed with BaP also identified alterations in sphingomyelins and glycerophospholipids (50), which was determined to be receptor independent and metabolically associated. BaP was also shown to alter cholesterol-rich micro domains, resulting in changed fatty acid composition and reduced cholesterol in rat liver epithelial cells. The data strongly suggests BaP exposure alters blood lipid composition, which was observed in APoE +/- knockout mice (51) and suggests the relationship is not due to binding and co-transport on lipoproteins alone.

Oxidative metabolic pathway associations with BaP included Cys and Met metabolism (Figure 7), an important component of central amino acid metabolism and antioxidant protection. Cys related intermediates include sulfopyruvate, cysteamine and hypotaurine. Cysteamine is a degradation product of cysteine, and acts as a precursor to hypotaurine, which in turn is oxidized to taurine. Hypotaurine and cysteamine are expected to function as protective antioxidants through oxidation of the sulfinic group (52, 53) through scavenging of OH, HOCl and H₂O₂. Relative expression levels of cysteamine and hypotaurine are in agreement with the two metabolites acting as protective antioxidants. Cysteamine was reduced in the high exposure group and negatively correlated with hypotaurine. Metabolism of BaP causes formation of a carbon-6 localized radical cation (54), which significantly contributes to DNA binding and adduct formation. It is also possible that the oxidation of cysteamine to hypotaurine occurred during storage; however, the high degree of enrichment within this pathway and dose dependent change with BaP suggests this behavior is due to biological response and not a sample storage artifact.

Met, a biochemical precursor to Cys, was elevated within the higher exposed group. Met is an essential amino acid, and a key metabolic precursor. Met is active in immune regulation, polyamine metabolism, DNA methylation (55) and glutathione (GSH) formation, which detoxifies BaP via GSH S-transferase catalyzed conjugation of BaP (56). Met has also been evaluated for a chemoprotective effect against BaP genotoxicity using HepG2 cells (57). Increased Met levels were found to reduce uptake of BaP while increasing discharge of BaP DNA adducts, up-regulating intracellular GSH and possibly altering protein expression contributing to formation. In our study, acetyl-Met, which acts as a bioavailable form of Met (58), was down-regulated within the higher serum level group. Previous studies have shown that acetyl-Met treatment can prevent liver toxicity by protecting against GSH depletion and stabilizing cellular methylation (59-61). Acetyl-Met is present and detectable within

mammalian tissues and body fluids (61), and the decreased level could arise due to a protective liver effect, a target organ for BaP toxicity.

Conclusion

The present study shows that DoDSR samples are of sufficient quality for chemical profiling of DoD personnel and identification of the BaP-associated metabolic perturbations. Insight into biological perturbations enables a basis for understanding possible health risks of environmental exposures and changes in metabolism that can result in manifestation of exposure-related disease pathophysiology. The ability of archived DoDSR samples to provide relevant biomarkers of exposure and effect make possible the assessment of exposures in military personnel while avoiding the additional burden and risk from the use of personal monitoring equipment.

While the lack of exposure data and small sample size limit the ability to generalize conclusions, the results show that the approach enables detection of metabolic alterations in humans that have either been observed experimentally in animal models or are physiologically plausible based upon existing knowledge. Thus, the data establish feasibility to use DoDSR serum samples for retrospective exposure surveillance. Future integration of this approach with well characterized, larger sample sizes will enable sequencing for both environmental surveillance and bioeffect monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
BaP serum concentrations in the thirty unidentified individuals. Quantified serum levels ranged from 0.13 ng/mL to 37.2 ng/mL, with an average concentration of 3.39 ng/mL.

Figure 2. Metabolite coverage of metabolic pathways following database matching to KEGG. Matches are represented by black dots. A total of 625 m/z features matched KEGG metabolites present within the human metabolic pathways, which included amino acid, lipid, steroid and xenobiotic metabolism.

Figure 3. Unsupervised hierarchical clustering heatmap of the top 100 SPLS significant features associated with BaP. Individual grouping was found to be strongly associated with exposure level (colored boxes on left axis), reflecting exposure-metabolic signatures within the chemical profile.

Figure 4. Network association of the sPLS m/z features with raw HRM data. Red circles represent sPLS selected features; green triangles are correlating features with Spearman $|r| \geq 0.6$ and FDR = 20%.

Figure 5.

Top 15 metabolic pathways with enrichment p -value from Mummichog using the sPLS and network associated features. Seven pathways were enriched (p -value ≤ 0.05 , filled bars) within the MWAS selected features.

Figure 6. Metabolite intensity differences from glycerophospholipid metabolism for individuals in the high and low BaP clusters.

Figure 7. Metabolite intensity differences from sulfur amino acid metabolism for individuals in the high and low BaP clusters.

Table 1
Serum concentrations of representative confirmed metabolites in the 30 unidentified samples

Metabolite	Detected m/z	Adduct	Average concentration $\pm$ SD (μ M)	Serum concentration range (μ M) ¹
Arginine	175.1194	+H	148 $\pm$ 39	130 $\pm$ 30 ^a
Glycine	120.0035	+2Na-H	280 $\pm$ 62	330 $\pm$ 106 ^a
Histidine	156.0771	+H	100 $\pm$ 12	143 $\pm$ 27 ^a
Ornithine	133.0974	+H	83 $\pm$ 28	93.8 $\pm$ 41 ^a
Phenylalanine	166.0867	+H	131 $\pm$ 18	78 $\pm$ 21 ^a
Threonine	120.0659	+H	136 $\pm$ 22	128 $\pm$ 41 ^a
Tryptophan	205.0974	+H	56 $\pm$ 7	55 $\pm$ 9.7 ^a
Tyrosine	182.0814	+H	84 $\pm$ 23	55 $\pm$ 9.7 ^a
Linoleic acid	281.2481	+H	2309 $\pm$ 157	2310-5190 ^b
α -Linolenic acid	279.2324	+H	54 $\pm$ 10	26-151 ^b
Glucose	203.0529	+Na	4310 $\pm$ 1153	4971 $\pm$ 373 ^a
Kynurenine	209.0925	+H	2 $\pm$ 0.4	2.4 $\pm$ 0.5 ^a
Carnitine	162.1128	+H	52 $\pm$ 9	30 $\pm$ 8 ^a
Creatinine	114.0667	+H	93 $\pm$ 13	87 $\pm$ 19 ^a
Creatine	132.0770	+H	16 $\pm$ 8	36.7 $\pm$ 28.3 ^a

^aWishart, D. S., et al. (2009). HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* **37**(Database issue): D603-610.

^bU.S. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012. Atlanta (GA): National Center for Environmental Health; April 2012. http://www.cdc.gov/nutritionreport/pdf/Nutrition_Book_complete508_final.pdf