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High-resolution metabolomics assessment of military personnel: Evaluating analytical strategies for chemical detection

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Abstract

Objective—To maximize detection of serum metabolites with high-resolution metabolomics (HRM).

Methods—Department of Defense Serum Repository (DoDSR) samples were analyzed using ultra-high resolution mass spectrometry with three complementary chromatographic phases and four ionization modes. Chemical coverage was evaluated by number of ions detected and accurate mass matches to a human metabolomics database.

Results—Individual HRM platforms provided accurate mass matches for up to 58% of the KEGG metabolite database. Combining two analytical methods increased matches to 72%, and included metabolites in most major human metabolic pathways and chemical classes. Detection and feature quality varied by analytical configuration.

Conclusions—Dual chromatography HRM with positive and negative electrospray ionization provides an effective generalized method for metabolic assessment of military personnel.

Introduction

Military personnel can be exposed to many environmental chemicals, and analytical methods which target specific chemicals or small groups of chemicals are inadequate to address the problem (1). High-resolution metabolomics (HRM) provides an affordable, high-throughput platform capable of advanced clinical chemistry measurements, environmental chemical surveillance and bioeffect monitoring. HRM relies on a combination of chromatography coupled to ultra-high resolution mass spectrometry and advanced

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computational approaches for spectral feature alignment, peak integration, and feature extraction (2–6). With this workflow, HRM is capable of reproducibly measuring greater than 10,000 unique spectral features [defined by a characteristic mass-to-charge ratio (m/z), retention time, and intensity] using small volumes of biological specimens. The resulting chemical profile, often referred to as the metabolic phenotype, includes a broad range of chemical classes and metabolic pathways (2–4, 6, 7). A number of studies have used HRM-based approaches to identify metabolic changes associated with a variety of disease, clinical or exposure settings, including Parkinson disease (8), pulmonary tuberculosis (9), HIV-1 infection (10), age-related macular degeneration (11), lung transplantation (12), alcohol abuse (13), and cadmium exposure (14). Furthermore, application of untargeted HRM approaches to population screening and clinical use provides improved capabilities for biomarker discovery and identifying unknown chemical exposures, with simultaneous measurements of metabolic network and pathway associations (15–17) for enhanced understanding of human health and disease.

Estimates of the metabolome range from 2,000 to >100,000 metabolites from endogenous sources (e.g., lipids, carbohydrates, nucleotides, amino acids, metabolic intermediates, signaling molecules, small peptides) and exogenous dietary and environmental sources (1, 5, 18, 19). The ability to detect the very large number of chemicals is complicated by the very broad range in abundance of chemicals in biologic systems, which span at least eight orders of magnitude in human plasma (7). Online chemical databases such as Metlin (over 240,000 entries), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (over 17,000 entries), or the Human Metabolome Database (HMDB) (over 40,000 entries) facilitate efforts to obtain unequivocal chemical identification of spectral features obtained from HRM analysis, but do not provide such identification alone. Despite this, databases provide useful resources to evaluate different analytical platforms, even without absolute identification. For instance, if one assumes that different platforms generate the same frequency of incorrect matches, then comparison of results provides an estimate of which platform gives better coverage. Similarly, the fraction of ions measured that do not have a match in the databases provides an estimate of the completeness of the databases. While imprecise, these approaches provide ways to compare performance of different platforms.

Chemicals present in the metabolome span a wide range of physicochemical properties (mass, polarity, abundance, lipophilicity, pKa), and numerous analytical strategies are available to analyze this complex mixture, including Nuclear Magnetic Resonance (NMR) or Fourier Transform Infrared Spectroscopy (FTIR) based methods, or mass spectrometric methods (MS) coupled to gas (GC), liquid chromatography (LC) or direct injection (DI) (6, 20–32). NMR and FTIR-based methods provide fast quantification of high abundance metabolites with minimal sample processing. Due to increased sensitivity enabling detection of low abundance chemicals, MS-based metabolomics provides improved capability for in-depth metabolic profiling.

Recent advances in MS technology, such as very high mass resolution (>60,000 resolving power), mass accuracy (<5 ppm), and increased scan speed of ultra-high resolution mass spectrometers, decreases the requirements for separation of chemicals prior to detection (33–35). Nonetheless, the use of liquid chromatography (LC) separation prior to MS improves

chemical coverage, sensitivity, and quantification, especially for complex biofluid analysis (6). Commonly used LC strategies include reverse phase (C₁₈) chromatography, hydrophilic interaction liquid chromatography (HILIC) and anion exchange (AE) chromatography. A variety of ion sources are also available for ionization of analytes prior to introduction into the mass spectrometer, including electrospray ionization (ESI) (36, 37) and atmospheric pressure chemical ionization (APCI) (28). Complementary ionization and separation approaches for metabolomics analyses have been applied for metabolic phenotyping studies of human populations. Dunn et. al (19), Want et. al (38), Rabinowitz et. al (27), Patti et. al (39), Psychogios et. al (40) and others have made considerable progress demonstrating the utility of different instrumentation. However, direct comparison of different chromatographic/ionization platforms for HRM is not available.

To identify the optimal analytical strategy for HRM profiling of serum obtained from military personnel, we analyzed a set of thirty non-identified serum samples obtained from the United States Department of Defense Serum Repository (DoDSR) with different combinations of HILIC, C₁₈, or AE chromatography and ionization strategies [positive (+) and negative (–) ESI and APCI]. We compared the total number of reproducible ions detected, defined as *m/z* features (accurate mass mass/charge with associated retention time and ion intensity) detected with each configuration and matches to known chemicals in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Human Metabolite database (41, 42). This strategy allowed us to perform an estimate of metabolic coverage of known and unknown chemicals based on the total number of ions detected and KEGG matches from common adducts. We followed this estimation by comparing detection of representative metabolites with each HRM strategy. This systematic evaluation provides guidance for optimal analytical configurations for metabolic phenotyping studies in military personnel.

Methods and Materials

Samples

Both Emory University and the USUHS IRBs reviewed and approved the research protocol as non-human subjects population health surveillance. Thirty unidentified serum samples (i.e., unknown source or date of collection) were obtained from the Department of Defense Serum Repository (DoDSR) for analysis. The repository consists of approximately 50 million serum samples originally collected for mandatory armed forces personnel HIV testing (43). The samples provided by the DoDSR for this study were from the long-term storage facility where samples are maintained at –30°C; the samples were shipped on dry ice to Emory University and maintained at –80°C until analysis. The protocols for collection and storage are described (43) and presumed to be consistent. Because associated records were not available, however, there is no way to verify that uniform procedures were followed.

Sample Preparation

An internal standard mixture consisting of 8 stable isotope internal standards was prepared in LCMS grade acetonitrile (Fluka Analytical). This mixture broadly represents different classes of small molecules for high-resolution metabolomics analysis. These chemicals

included [$^{13}\text{C}_6$]-D-glucose, [^{15}N]-indole, [$^{13}\text{C}_5$]-L-glutamic acid, [^{15}N]-L-tyrosine, [trimethyl- $^{13}\text{C}_3$]-caffeine, [3,3- $^{13}\text{C}_2$]-cystine, [^{15}N , $^{13}\text{C}_5$]-L-methionine, and [$^{13}\text{C}_5$, $^{15}\text{N}_2$]-L-glutamine. All internal standards were obtained from Cambridge Isotope Laboratories and >98% pure, according to the manufacturer's certificate of analyses. Accurate masses for the "M+H", "M+Na", or "M-H" adducts for each internal standard compound were used to verify the presence of standard in each sample. Each sample was prepared according to Soltow et al. (6) by adding 130 μL of acetonitrile containing the internal standard mixture to 65 μL of serum. Following mixing and incubation on ice for 30 min, precipitated proteins were pelleted with 10 min centrifugation at $16,100 \times g$ at 4°C . The resulting supernatant was transferred into autosampler vials and maintained at 4°C for the duration of the analysis (<24 hours). Pooled plasma reference samples (Qstd) and the NIST SRM 1950 certified pooled plasma standard reference material (44) were prepared and included at the beginning and end of each batch of 15 samples.

Liquid chromatography

Chromatographic separation was performed on a Dionex Ultimate 3000 UHPLC with a dual column compartment for column switching. This setup allowed an analytical separation to be performed on one column while a second column was washed and conditioned prior to the next injection. For each set of analyses, a single chromatographic retention mechanism was employed, with the ionization polarity alternating between injections. Mobile phase A consisted of 2% formic acid (Sigma-Aldrich, analytical grade 27001) in LCMS grade water (Fluka Analytical Chromasolv LCMS grade). Mobile phase B consisted of LCMS grade acetonitrile, and mobile phase C consisted of LCMS grade water. Following each analytical injection, each column was washed and reconditioned at the starting mobile phase conditions for 20 min. The flow rate for all analytical separations was 350 $\mu\text{L}/\text{min}$, injection volume was 10 μL , and all samples were analyzed in triplicate, allowing averaging of three analyses to enhance reproducibility. Although we commonly use run times of 10 min for LCMS of plasma, we used 20 min gradients in this study to see if higher numbers of ions or better quality was obtained; no substantive differences were apparent (data not shown).

Reverse Phase (C_{18}) chromatography

Higgins C_{18} 100 $\times$ 2.1 mm (TS-1021-C185) columns were used for reversed phase separation. For C_{18} /APCI or ESI+ analysis, the initial mobile phase conditions consisted of 5% A, 15% B, and 80% C for four min. This was followed by a 10 min linear gradient to 5% A and 95% B, which was then held for 6 min. For C_{18} /APCI or ESI- analysis, the initial conditions were 80% A, 15% B, and 5% C for 4 min, increased to 95% B and 5% C for 10 min, which was then held for 6 min.

Anion Exchange (AE) chromatography

Hamilton PRP-X110 100 $\times$ 2.1 mm (79743) columns were used for AE chromatography. For AE/ESI+ and ESI- analysis, the initial mobile phase conditions consisted of 5% A, 50% B, and 45% C held for 2 min, increased to 50% A, and 50% B for min and held for 13 min.

Hydrophilic Interaction Liquid Chromatography (HILIC)

Supelco Ascentis Express HILIC 100 × 2.1 mm mm (53939-U) columns were used for HILIC chromatography. For HILIC/ESI+ analysis, the initial mobile phase conditions consisted of 8% A, 90% B, and 2% C, held for 4 min, increased to 50% A, 45% B, and 5% C for 10 min, and held for 6 min. For HILIC/ESI– analysis, the initial mobile phase conditions consisted of 98% B, and 2% C, held for 4 min, increased to 5% A, 45% B, and 50% C for 10 min and held for 6 min.

Ultra-high resolution mass spectrometry

Mass spectral detection was performed with a Thermo Scientific Q-Exactive HF mass spectrometer in continuous full scan mode at 70,000 resolution (scan range 85–1,275 m/z for all analyses other than AE, AE scan range was 100–1,500 m/z). This difference in mass range resulted in approximately 200 fewer features detected by omission of 85–100 m/z , and 500 more features detected due to inclusion of 1,275 to 1,500 m/z . Ion source conditions were optimized for both negative and positive ionization through systematic variation of different operational parameters to maximize the signal intensity of a representative chemical mixture infused into the source at appropriate mobile phase flow rate and composition. The automatic gain control (AGC) target was set at 10^6 with a maximum ion injection time at 200 ms. Positive mode conditions were: spray voltage, 4500 V; capillary temperature, 275°C. Negative mode conditions were: spray voltage 3200 V; capillary temperature, 320°C. For both modes, sheath and auxiliary gas flow rates were maintained at 45 and 5 (arbitrary units), respectively. The S-Lens RF level was set at 69 for both negative and positive mode.

Data extraction and analysis

Instrument .RAW files were converted to .CDF format and mass spectral features were extracted and aligned using apLCMS (2, 4) with modifications by xMSanalyzer (3). The apLCMS software includes baseline subtraction, noise filtering (based upon a feature being present in at least 10% of spectra), retention time alignment (30 second maximum drift allowed) (2). xMSanalyzer was used with default parameter settings, and all samples met quality control criteria for mass alignment of internal standards, total feature detection and reproducibility of replicates. To further ensure analytical reproducibility and minimize measurement variability (assessed by ion technical replicate CV), a feature was required to be detected on at least two out of three technical replicates, and features with greater than median 50% CV for technical replicates were removed from subsequent analyses. To estimate the number of chemicals detected in a single analysis, we performed tentative metabolite annotation by using the *feat.batch.annotation.KEGG()* function in xMSanalyzer using an m/z search tolerance of ± 10 ppm and “M+H”, “M+Na”, “M+K”, “M-H₂O+H”, “M+ACN+H”, “M+ACN+Na”, “M+2Na-H” adducts for positive mode; “M-H”, “M+Cl”, “M-H₂O-2H”, and “M+Na-2H” adducts for negative mode. The 10 ppm window is based on previous studies showing that even though most ions are within 5 ppm mass accuracy, the apLCMS data extraction algorithm can result in greater differences between observed and exact mass (2, 4, 6). We also annotated metabolites with a conservative approach by only searching for “M+H” ions in positive mode or “M-H” ions in negative mode. In both cases,

we minimized redundancies by eliminating duplicate KEGG Compound IDs. We mapped unique chemical matches onto metabolic pathways using the KEGG mapper tool accessed at http://www.genome.jp/kegg/tool/map_pathway2.html. The online *Bio Venn* tool (<http://www.cmbi.ru.nl/cdd/biovenn/>) was used to compare unique and overlapping chemical matches and ions detected by different HRM strategies (35).

Results

Characteristics of HRM data

Supplement 1 contains histograms of feature triplicate median coefficient of variation (CV), distribution of m/z as a function of retention time (RT), and ion detection as a function of RT for each mode of chromatography and ionization. All platforms except C₁₈/APCI+ had median CV < 30%, equivalent to Standard Error of the Mean (SEM) <17.4%, indicating large numbers of ions are detected with sufficient reproducibility for health evaluation (Supplement 1, left panels). The distribution of m/z as a function of RT provides a way to visualize elution profiles. These results (Supplement 1, middle panels) show that metabolites are differentially retained depending on the choice of AE, C₁₈, or HILIC chromatography and that ion elution profiles are similar for positive and negative ionization modes. Histograms of ions detected as a function of RT (Supplement 1, right panels) show that maximal ion detection occurs during the initial wash-through volume, likely due to the salt content of plasma. For both C₁₈ and HILIC, subsequent peaks were also consistent with the known mixture of hydrophobic and hydrophilic metabolites in plasma.

Total number of m/z features detected and KEGG matches for individual HRM platform

The total number of ions detected for each analysis platform varied from 17,824 for C₁₈/ESI – to 2,559 for C₁₈/APCI+ (Figure 1A). Analytical configurations using ESI detected more ions than configurations using APCI. Negative ionization resulted in a greater number of detected ions than the comparable analyses with positive ionization (Supplement 2), except for AE: HILIC/ESI– (16,777) versus HILIC/ESI+ (13,404); C₁₈/ESI– (17,824) versus C₁₈/ESI+ (10,722); C₁₈/APCI– (7,043) versus C₁₈/APCI+ (2,559); AE/ESI+ (9,931) versus AE/ESI– (8,182). To estimate the number of ions which possibly represent known metabolites, we searched the KEGG database for chemical matches corresponding to the adduct forms described above. The results showed that only the configurations with relatively lower number of detected ions had more than 50% database matches, suggesting that many detected ions could be derived from uncharacterized metabolites.

To estimate the coverage of known metabolites provided by each configuration, we calculated the percentage of the KEGG chemical database (17,554 total chemicals) matched by at least one ion in each method. The results (Figure 1B) showed that if all matches were correct, the best platforms (HILIC/ESI+ and AE/ESI+) could detect up to 58% of the KEGG chemical database. In combination with the results in Figure 1A, indicating the majority of detected ions are not matched to chemicals in KEGG, these results suggest the KEGG database is incomplete in coverage of the human metabolome and that a single analytic platform captures less than 58% of metabolites in the database.

Dual HRM platforms to increase metabolic coverage

To determine the extent that dual HRM strategies could improve the number of database matches, we examined pairwise combinations of ionizations and chromatography. Similar total ion count was obtained with any paired combination of positive and negative ESI using either HILIC or C₁₈ chromatography. To determine combinations that provided the maximum number of database matches, we examined paired LC and ionization configurations using number of non-overlapping and overlapping matches to the KEGG database. The pair consisting of HILIC/ESI⁺ and C₁₈/ESI[−] analysis had the highest number of chemical matches (12,712), which included matches to 72% of the chemicals present in the KEGG database (Figure 2; Supplement 3). A number of dual HRM strategies achieved greater than 70% coverage of the KEGG database. For example, using HILIC chromatography with dual polarity (ESI⁺ and ESI[−]) provided 12,550 matches. Using C₁₈ chromatography with ESI⁺ and ESI[−] resulted in a total of 12,454 matches. The combination of C₁₈/ESI⁺ with HILIC/ESI[−] matched to 12,271 chemicals in KEGG. Thus, either of these dual HRM platforms appears to provide an effective way to increase the number of database matches by approximately 25% over individual HRM strategies.

The use of ESI/APCI or C18/HILIC/AE increases ion detection for HRM

To determine which pair of columns provided maximal number of ions with matches to the KEGG database, we compared the effect of chromatography on the detection of ions matching at least one chemical with comparisons for positive and negative ionization completed separately (Figure 3, Supplement 4). Direct comparisons of ions obtained in negative mode with ions obtained in positive mode are not possible unless molecular ions for chemicals are identified. Therefore, this analysis was to compare the effect of chromatography on the ability to detect unique ions that could match chemicals in KEGG. The use of different columns resulted in a relative increase in non-redundant ions, i.e., likely increasing the total number of chemicals detected. C₁₈/ESI[−] detected 3,642 ions with database matches and HILIC/ESI[−] detected 4,578 ions with database matches, with only 975 of these matches being the same for the two platforms. Similar increases in the number of ions with at least one database match were observed when any two chromatographic strategies were used. Thus, the data show that in a dual platform analysis, the use of two different column types improves chemical coverage.

In comparison to the large overlap in the number of chemical matches obtained for HILIC/ESI⁺ and C₁₈/ESI⁺ (68% of ions have *m/z* within 10 ppm), only 13% of ions with database match for at least one chemical were the same for HILIC/ESI⁺ and C₁₈/ESI⁺. These results show that detected ions with chemical matches are different depending on the HRM configuration, and that employing multiple analytical approaches can increase the detection of ions with chemical matches. Furthermore, these data suggest that the chemicals detected by both platforms were high abundance, present in multiple adduct forms and potentially suitable for internal cross-validation within a dual-chromatography protocol (6). Support for this concept was provided by comparison of the signal for tyrosine in the HILIC/ESI⁺ and C₁₈/ESI⁺ comparison (Figure 4).

HRM detected endogenous and exogenous chemicals

Combining all eight HRM platforms resulted in 14,387 different database matches, providing up to 82% coverage of all metabolites in KEGG. KEGG matches were then classified based on their functional KEGG BRITE categorization (Fig 5). Approximately half of KEGG matches did not have KEGG classifications. Metabolites with roles in intermediary metabolism are termed “compounds with biological roles” and represented 3.4% of all KEGG matches while lipids represented a larger fraction, 12.3%. The remaining matches were for chemicals derived from exogenous sources (pesticides, carcinogens, pharmaceuticals, phytochemical compounds, endocrine disrupting compounds, natural toxins, and metabolites derived from natural products). These results show that diverse classes of chemicals were detected in a single analysis, including those derived from environmental or occupational exposures.

Pathway Coverage

To determine the coverage of metabolic pathways provided by dual chromatography, we mapped the database matches to KEGG human metabolic pathways. Combining C₁₈/ESI⁻ with HILIC/ESI⁺ (Figure 6) analysis provided similar coverage (1246 database matches) of the human reference metabolic pathway in KEGG (hsa01100) as C₁₈/ESI⁻ with C₁₈/ESI⁺ and HILIC/ESI⁻ with HILIC/ESI⁺ (data not shown). In previous targeted MS/MS confirmation of database matches to ions detected by comparable methods, we found that 60% to 80% of these matches were correct identifications (3, 6, 45). Thus, with recognition that confirmation of metabolite identity is needed for specific conclusions, the results show that a dual chromatography approach provides effective coverage of central metabolic pathways.

We performed a comparable annotation using only ions matching [M+H] or [M-H] adducts to determine the utility of this simpler annotation strategy. Results for HILIC/ESI⁺ and C₁₈/ESI⁻ showed 757 matches to chemicals on hsa01100 were obtained, with less overlapped detection between platforms (Figure 7). Despite the lesser number of matches, this simpler strategy provided similar coverage of metabolic pathways. Although this more conservative strategy still includes some incorrect matches, the analysis emphasizes that combined platforms are best to maximize metabolic coverage.

Comparison of Targeted Chemical Detection

The information provided by the analyses above suggests that the combination of different polarities with different analytical columns provides optimal chemical coverage. To test this specifically, we assessed each HRM configuration for its ability to detect a list of metabolites with confirmed identities (Figure 8; Supplement 5). [3,3-¹³C₂]-cystine, an internal standard, was detected in seven of the eight platforms. In contrast, cotinine, was only detected with positive ionization (177.1026 *m/z*) and fatty acids were better detected with negative ionization. The results show that inclusion of both polarities within a dual chromatography analysis improves the coverage of known metabolites.

Discussion

Management of risks associated with environmental exposures and their biologic effects depend upon tools to detect and predict adverse effects. Currently used clinical chemistry and toxicological analyses provide information about a limited number of markers, and there is an ongoing need to improve capabilities. HRM provides a strategy to measure thousands of known and unknown chemicals to enable routine, generalized assessment of exposures and biologic responses. These metabolic assessments can be used within an integrative framework for health evaluation in military personnel and support National Precision Medicine initiatives. In this study, we sought to identify HRM configurations that could increase ion detection and chemical database matches, and thereby increase the likelihood of detecting the maximal number of known and unknown chemicals. We evaluated different chromatographic and ionization strategies for analyzing serum samples from the DoDSR to identify optimal strategies for metabolic assessments of military personnel.

Chromatography facilitates the analysis of complex mixtures using mass spectrometry, as it separates potential interfering ions from ions of interest and improves quantification and limit of detection. Orthogonal dual chromatographic approaches prior to MS analysis, such as pairing lipophilic selectivity obtained with C₁₈ chromatography with polar selectivity obtained from either AE or HILIC analysis, have been applied in previous studies. In Soltow et al. (6), the use of dual AE and C₁₈ chromatography with positive ionization on a Thermo LTQ-FT mass spectrometer increased overall ion detection by 23–26%, yielding a total of up to 7,000 ions per sample (6). More recently, Ivanisevic et al. (24) performed a similar analysis of human plasma, comparing chemical detection with C₁₈ and HILIC using positive and negative electrospray ionization and obtained 9,709 ions with HILIC/ESI+ and 15,263 ions with C₁₈/ESI+. In negative mode, they obtained 8,122 ions with HILIC/ESI– and 7,742 ions with C₁₈/ESI–. Our HRM platform yielded similar numbers of *m/z* features detected with HILIC /ESI+ (13,404 ions) and C₁₈/ESI+ (10,722) and with HILIC/ESI– (17,824) and C₁₈/ESI– (16,777 ions). The Ivanisevic study also demonstrated that the combination of HILIC/ESI+ with C₁₈/ESI+ increased ions with [M+H] chemical matches by 28%, compared to using C₁₈/ESI+ alone. In negative mode, the combination of HILIC/ESI– with C₁₈/ESI– increased ions with [M-H] chemical matches by 43%, compared to using C₁₈/ESI– alone. Contrepois et. al (46) also compared ions detected by HILIC and C₁₈ chromatography with positive and negative ESI, and found that the addition of HILIC chromatography to a C₁₈ metabolomics analysis resulted in a 68% increase in ions detected with ESI+, and a 148% increase in ESI–. In our study, we found that pairing HILIC chromatography with C₁₈ chromatography for HRM increased detected ions with chemical matches by 99% using ESI–, and a 90% increase using ESI+, compared to using C₁₈ chromatography alone. Our findings are consistent with other studies evaluating combined analytical strategies for metabolic profiling, showing that dual chromatographic approaches increase the number of ions detected with non-redundant chemical matches.

Metabolites may preferentially form positive or negative ions for detection, and may ionize more efficiently using either ESI or APCI. Because producing gas phase ions from metabolites is critical for mass spectral detection, we also examined the use of different ionization strategies (ESI vs. APCI, ESI+ vs. ESI–) for increasing potential chemical

coverage for HRM. Previous studies have performed similar comparisons for metabolomics. Nordström et al. showed that pairing ESI+ with ESI- increased unique, non-redundant ion detection by 90% compared to using ESI+ alone, and that the use of APCI increases unique ion detection by 20% compared to ESI alone (28). Ivanisevic et al. showed that the combination of HILIC with ESI- and C₁₈ with ESI+ provided the maximum amount of biological information relating to lipid and central carbon metabolism (24). Contrepolis et al. also compared HILIC and C₁₈ chromatography with positive and negative ESI and demonstrated the use of dual chromatography with positive and negative ionization increased chemical detected (46). In our study, we show similar increases in chemical coverage when using positive and negative ionization for HRM. In contrast to our extraction with a volume of 2:1 acetonitrile to plasma and analysis with an acidic pH, Ivanisevic et al. extracted with a volume of 1:1 methanol/acetonitrile to plasma and the chromatographic gradients were buffered at basic pH; a direct comparison of extraction and solvent conditions, as well as column stability, will be needed to determine which conditions are most suitable for routine use.

The number of database matches provides an estimate of metabolic coverage. As indicated above, targeted MS/MS confirmation of database matches in our previous studies showed that between 60% and 80% of matches are correct identifications (3, 6, 45). Other limitations to interpretation, namely that a single chemical can give rise to multiple adducts and that multiple chemicals have the same mass, were addressed in the experimental design. Specifically, we estimated the fraction of ions with putative identification by counting all ions with matches, and we eliminated redundancies due to multiple adducts of the same chemical by counting database matches in terms of the number of KEGG IDs. The limitations were also addressed by analysis of a subset of metabolites with confirmed identities (Fig 8). Thus, despite the limitations of the approach, the results provide rough estimates that about half of the KEGG database can be captured in routine analyses and that about half of the ions detected are present in the KEGG database.

Analysis of matches using only H+ and H- forms showed that even with this simplification, an effective coverage of nearly 1000 metabolites was obtained. Confirmation of these metabolites by MS/MS for H+ and H- forms is straightforward and, along with reference standardization (7), could provide a way to quantify up to 1000 metabolites in a routine and affordable manner. Furthermore, this approach could allow integration of data from ultra-high resolution instruments and data from Q-TOF and other instruments so that data are readily interchangeable between different laboratories.

Contrepolis et. al noted that while chemicals may be detected by multiple analytical configurations, peak quality may vary based on differences in ionization or chromatography. We noted in our study that different HRM strategies resulted in differential detection of representative chemicals. Overall, our study results are in agreement with previous studies and others (20, 23,29,38,47–50), as we conclude that employing dual HRM with positive and negative ionization and orthogonal chromatography increases chemical coverage of human serum metabolites and that the quality and quantification of detected chemicals may vary depending on HRM conditions. Increased chemical coverage facilitates the use of non-

targeted metabolomics pathway enrichment software i.e. *mummichog*, which performs metabolic pathway enrichment analysis from ranked spectral features (17).

Limitations of the Study

The computational metabolomics methods used in the present study provide estimates of coverage of metabolism and detection of dietary and environmental chemicals based upon accurate mass matches to chemicals in the KEGG database. A limitation is that these analyses do not provide absolute chemical identity for most of the ions detected. Confirmation of chemical identities with tandem mass spectral (MS/MS) analysis and co-elution with authentic standards has been performed for several hundred chemicals in other studies using these methods (7–12,14,16,45, 51)) and provide confidence that the conclusions reached are valid, even though many individual ion matches are incorrect.

In addition to confirming chemicals with tandem mass spectrometry, future studies can explore combining NMR/GCMS technologies to increase chemical detection (such as performed by Psychogios et. al (40)), optimization of analytical gradients or the use of ion mobility MS to increase chemical coverage, and application of spectral de-convolution software such as CAMERA (52) or xMSannotator (K Uppal, DI Walker, DP Jones, unpublished) to identify isotopes and adducts associated with molecular ions. Application of complementary analytical methodologies and spectral de-convolution software have greatly increased chemical detection, the confidence of chemical matches and reduced chemical noise and artifacts from MS analysis (22, 46, 52, 53).

Conclusion

This study compared chemical coverage obtained with eight different HRM platforms. The results show that orthogonal chromatography and polarity for ionization (e.g., HILIC/ESI+ and C₁₈/ESI–) provides the best dual chromatography platform for metabolic coverage of serum metabolites and also includes an extensive number of matches to dietary and environmental chemicals. The results show that metabolites detected by multiple platforms can be used for internal validation and that little pathway information is lost by restricting analysis only to adducts formed by gain or loss of H⁺. The results support the use of HRM with DoDSR serum samples for metabolic assessments of military service personnel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Total number of ions detected with percentage of ions matching at least one chemical in KEGG represented by dark portion of the bar. B. Number of chemical matches, with percentage of total database (17,554 chemicals) matched.

Figure 2. Dual HRM platforms increase number of database matches to 72% of the KEGG chemical database. Matches obtained from left HRM platform (blue), right HRM platform (red), and both HRM methods (purple).

Figure 3. Number of ions with database matches from paired HRM platforms. Ions detected by left HRM platform (blue), right HRM platform (red), and both HRM methods (purple).

Tyrosine

Figure 4.
Comparison of ion intensity for tyrosine detection by HILIC/ESI+ and C₁₈/ESI+.

Figure 5.
HRM detects metabolites from a broad range of chemical classes.

Figure 6.

Dual HRM using C_{18}/ESI^- (blue) with HILIC/ ESI^+ (red) provides in-depth coverage of human metabolic pathways using multiple adducts for chemical matching. Black dots indicate metabolites matched with both HRM methods. 1246 metabolites are matched with this strategy.

Figure 7.

Dual HRM using C₁₈/ESI⁻ (blue) with HILIC/ESI⁺ (red) provides adequate coverage of human metabolic pathways using conservative chemical matching strategy. Black dots indicate metabolites matched with both HRM methods. 757 metabolites are matched.

Figure 8.
Representative chemical detection with eight HRM platforms.