Published in final edited form as:

Rapid Commun Mass Spectrom. 2016 November 15; 30(21): 2341–2350. doi:10.1002/rcm.7726.

Nontargeted analysis of the urine nonpolar sulfateome: a pathway to the nonpolar xenobiotic exposome

Yuanyuan Yao[‡], Poguang Wang[‡], Gang Shao[‡], Liza V. Anzalota Del Toro[±], Jose Codero[±], and Roger W. Giese^{‡,*}

[‡]Northeastern University, Department of Pharmaceutical Sciences and Barnett Institute, Bouve College, 360 Huntington Ave. Boston, MA 02115 USA

[±]University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico

Abstract

RATIONALE—Testing the urine nonpolar sulfateome can enable discovery of xenobiotics that are most likely to be bioactive. This is based on the fact that nonpolar xenobiotics are more likely to enter cells where they tend to undergo metabolism, in part, to sulfates that are then largely excreted into the urine.

METHODS—The following sequence of steps, with conditions that achieve high reproducibility, was applied to large human urine samples: (1) competitive nonpolar extraction with a porous extraction paddle; (2) weak anion exchange extraction with strong organic washing; and (3) UHPLC/negative ion-MALDI-TOF/TOF-MS with recording of ions with S/N > 20 that yielded M-1-80 (loss of SO₃) or *m/z* 97 (HSO₄[−]) upon fragmentation.

RESULTS—From a collection of urine samples from six pregnant women, the masses of 1129 putative sulfates were measured. Three lists of candidate compounds (preliminary hits) from these masses were formed by searching METLIN, especially via MATLAB, yielding putative xenobiotic contaminants (35 compounds), steroids (122), and flavonoids (1582).

CONCLUSION—A new way to reveal some of the nonpolar xenobiotic exposome has been developed that applies to urine samples. The value of the method is to suggest xenobiotics for subsequent targeted analysis in the population of people under study, in order to relate the environment to health and disease.

Keywords

xenobiotic exposome; mass spectrometry; nontargeted analysis; sulfateome; urine

Correspondence to: Roger W. Giese, Northeastern University, 206 The Fenway, 360 Huntington Ave. Boston, MA, USA 02115, r.giese@neu.edu.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

INTRODUCTION

The goal of this project was to set up a method, based on mass spectrometry, with broad scope and high reproducibility for detection of the urine nonpolar sulfateome. In part, such a method is important to provide a pathway to some of the urine xenobiotic exposome. It is challenging to measure the urine xenobiotic exposome in a nontargeted way, largely because of the high numbers and levels of normal metabolites in urine, coupled with the need to minimize the specificity of sample preparation in order to cast a broad net for xenobiotics.

In an accompanying paper^[1] we report a solid phase extraction device termed a “porous extraction paddle (PEP)” for extracting a large volume of urine at a remote location where resources are limited. Here we apply this technique to six urine samples collected from pregnant women in Puerto Rico, towards a long term goal of discovering xenobiotics that contribute to preterm birth. Some targeted studies have reported a correlation between xenobiotics and this pregnancy outcome, for the following compounds: bisphenol A,^[2] phthalates,^[3] polybrominated diphenylethers,^[4] and polychlorinated biphenyls and dioxins.^[5]

While most studies of urine sulfate conjugates have been conducted by targeted analysis, a few nontargeted studies have been reported. LC/ESI-MS was employed to analyze sulfate conjugates in both human and rat urine.^[6] Three negative ion detection modes were employed: constant loss of 80 u (SO_3^-); precursor ion scanning based on monitoring m/z 80 (SO_3^-); and precursor ion scanning based on monitoring m/z 97 (HSO_4^- , but this can also be H_2PO_4^- at low resolution). Each detection mode was reported to furnish about 10–15 peaks; few peaks were identified; and, based on retention times and the different detection modes, the peaks were reported to arise from different compounds for the human and rat urines.

In a general metabolomics study of human urine by LC/Orbitrap mass spectrometry, with a focus on detection and annotation of polar metabolites, 13 sulfates and 1 glucuronide were identified along with many other compounds.^[7] In another general metabolomics study of human urine, 5 sulfates and 9 glucuronides were identified along with many other compounds.^[8] Related work in the mode of targeted analysis also has been conducted for urine sulfates. For example, sulfate conjugates of five androgens were quantified in human urine by LC/ESI-MS; ^[9] also corresponding glucuronides were quantified by GC/MS in this study. Seven unconjugated, 19 glucuronide, and 10 sulfate forms of anabolic steroids in human urine were measured by LC/ESI-MS.^[10] Others^[11] used a combination of SPE, NMR and MS techniques to analyze human urine for phenolic conjugates (mostly glucuronides) after tea intake, achieving annotation of 138 and identification of 36 such compounds.

Here we report a method with some similarity especially to the first one cited above^[6] since we rely on characteristic fragmentation properties of sulfate conjugates to screen for their detection in urine. However our analysis is based on MALDI rather than ESI mass spectrometry, and we prepare the samples differently. Overall we report the detection of masses that correspond to 1129 nonpolar putative urinary sulfate conjugates after testing urine samples from six pregnant women in Puerto Rico. We also present some lists of

compounds having masses that correspond to the ones detected, and thereby suggest the identities of some of these compounds.

EXPERIMENTAL

Materials and methods

Eppendorf tubes (1.5 mL, Cat. No. 05-408-129), and 15 mL disposable centrifuge tubes (Cat. No. 05-539-4) were purchased from Fisher Scientific (Waltham MA, USA). Polymeric weak anion solid phase extraction (SPE) cartridges (bed size: 200 mg/ 3 mL; Part. No. 8B-S038-FBJ) were purchased from Phenomenex (Torrance, CA, USA). HPLC vials (250 μ L, Cat. No. 9301-0977) were purchased from Agilent (Santa Clara, CA, USA).

Triethylammonium acetate buffer (TEAA, 1.0 M in water at pH 7.0), ammonium acetate (99.999%), ammonium hydroxide water (29.9%), trifluoroacetic acid (TFA, 99%), α -cyano-4-hydroxycinnamic acid (CCA, 99.0%), and ammonium phosphate dibasic (99.99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water (Cat. No. 7732-18-5) and HPLC grade acetonitrile (ACN, Cat. No. 75-05-8), and 5 mL Cryovare Vials (Nalgene 5000-0050) were from Thermo Fisher Scientific (Waltham, MA, USA). Elution Solution A was made by mixing 300 μ L ACN, 700 μ L DI (deionized) water, and 110 μ L TEAA. Elution Solution B was made by mixing 800 μ L ACN, 200 μ L DI water, and 110 μ L TEAA. Reconstitution Solution C was made by mixing 10 μ L ACN, 990 μ L DI water, and 110 μ L TEAA.

Urine collection and extraction

Urine (1.8 L) was collected under IRB approval as accumulated first morning voids (kept dark) over a week from each of six pregnant women in Puerto Rico, preserved with acetic acid (4% final concentration; initially the collection jar contained 200 mL of 40% acetic acid) and subjected to solid phase extraction with 2.0 g of solid phase extraction particles comprising equal amounts of bonded silicas (strong anion exchange, strong cation exchange, C18, phenyl), a polyamide, and a hydrophilic-modified styrene polymer. These particles as a mixture were contained in a rigidified porous nylon bag designated as a “porous extraction paddle or PEP,” as described.^[1]

The rigidified, urine-exposed PEP bags were kept at -20° C in Puerto Rico in a FEP bag until overnight shipment on dry ice to Northeastern University in Boston, MA, USA for chemical analysis. Upon receipt in Boston, the FEP bags were stored at -80° C until processing as follows: (1) stirred a PEP in 2 L of water for 1 h^[1]; (2) recovered the nylon bag from the PEP, blotted excess solvent gently with a Kimwipe, and dried in a jar over Drierite, and (3) cut the bag open and transferred its contents into a 5 mL Cryovare Vial (pre-rinsed with methanol). The vial was gently inverted 20x to thoroughly homogenize the particles, and stored at -80° C.

Elution of urine-exposed, solid phase extraction particles

From a 2.0 g of extraction particles, a 30 mg aliquot was added to an Eppendorf tube (1.5 mL), followed by 0.5 mL of Elution Solution A. After shaking on a pulsing vortex mixer (Cat. No. 02215375, Thermo Fisher Scientific) for 30 min, at a speed of 1750 RPM, the

sample was rotary-vortexed and then centrifuged in a Marathon Micro H (Fisher Scientific, Waltham, MA, USA) at speed level 7 for 1 min. With an Eppendorf pipette, 450 μ L of supernatant was transferred to another Eppendorf tube and centrifuged similarly for 1 min. Supernatant (400 μ L) was taken into another Eppendorf tube, giving Supernatant 1. To the above Eppendorf tube containing residual particles was added 0.5 mL of Elution Solution B followed by shaking, vortexing, centrifugation and transferring as above, yielding 400 μ L of Supernatant 2. Supernatants 1 and 2 were combined in a 15 mL disposable centrifuge tube and the total volume was brought to 4 mL with DI water, yielding Combined Supernatant 3.

Weak anion exchange extraction

A Phenomenex Strata X-AW 33 μ m polymeric weak anion cartridge (200 mg/3 mL) was conditioned by gravity with 2 mL methanol (takes about 12 min), followed by 2 mL of DI water (takes about 21 min). Combined Supernatant 3 (4 mL) was loaded 2 mL at a time onto the cartridge (takes about 20 min/2 mL). The cartridge was washed with 4 mL of 25 mM ammonium acetate (takes about 30 min/2 mL), followed by 4 mL of methanol (takes about 22 min/2 mL). The cartridge was eluted with 2 mL of 5% ammonium hydroxide in 50% methanol (takes about 50 min). The elution fraction was transferred to two Eppendorf tubes (1 mL elution solution/tube) and evaporated in a Speed Vac (Savant Instruments, Saroor Nagar, Hyderabad, India; the manufacture-specified vacuum was 0.6 Torr) at room temperature for 2.5 h. The resulting solid was reconstituted with 250 μ L of Reconstitution Solution C followed by centrifugation as above. With an Eppendorf pipette, 200 μ L supernatant (Urine Extract) was transferred into a 250 μ L HPLC vial, and stored at -20°C prior to loading into an autosampler for LC/MS analysis.

Liquid chromatography with column switching and droplet collection

This was done on a Thermo Scientific Dionex UltiMateTM 3000 RSLC nano system (Sunnyvale, CA, USA). The analytical column was a capillary Acclaim PepMap RSLC (Dionex, Sunnyvale, CA, USA) C18 column (300 μ m $\times$ 15 cm, 2 μ m) that was kept at 30°C and operated at a flow rate of 4 μ L/min. The guard/trap column was a C18 PepMap 100 μ -precolumn (300 μ m $\times$ 5 mm, 5 μ m). Mobile phase A was water with 0.05% TFA and mobile phase B was acetonitrile. The trap column mobile phase was 1% acetonitrile with 20 mM TEAA. The samples were maintained at 5°C in the autosampler. The Urine Extract (5 μ L) was injected and the mobile phase was as follows: 4 min with 20 mM TEAA / 1% acetonitrile at 20 μ L/min on the trap column (sent to waste), followed by a linear gradient after switching from 10% to 80% solvent B in 100 min, held at 80% of B for 4 min, returned to 10% B in 1 min, and re-equilibrated at 10% B for 3 min. UV absorbance detection was monitored at 220 nm, 260 nm and 320 nm. Collection of the eluate as 20 sec droplets was done with a Probot Microfraction Collector (Dionex) onto a blank MALDI plate (Opti-TOF 384 Well Insert, 123 $\times$ 81 mm, AB SCIEX, Framingham, MA, USA). CCA matrix (0.5 μ L of 5 mg/mL in 50% acetonitrile with 7 mM ammonium phosphate dibasic to suppress salt adducts) was added to each spot manually using an Eppendorf Research Pro electronic pipette.

Mass spectrometry

MS and MS/MS analysis of the spots on the MALDI plate were performed using a AB SCIEX TOF/TOF™ 5800 system. After data acquisition on each LC/MALDI sample spot in the MS mode, fractionation of automatically selected precursors (precursor selection criterion: S/N > 20; 250 m/z to 850 m/z ; CCA matrix and adduct ions excluded with a tolerance of ± 0.01 m/z units; maximum of 100 precursor ions from each spot) was performed in a negative 1 kV mode with air ($\sim 1 \times 10^{-6}$ Torr) as the collision gas. The original MS data, stored in the TOF/TOF data base, was processed with Peak Explorer (AB Sciex).

RESULTS AND DISCUSSION

Analytical scheme: introduction

In our overall method for the human urine nonpolar sulfate conjugateome, there are two stages, as summarized in Figure 1. In the first stage (steps 1–4), a person furnishes a large volume of urine (about 2 L including acetic acid as a preservative) as an accumulation of first morning voids over a period of one week for mainly two reasons: to help in defining an average exposure, and to provide sufficient sample for multiple and scaled-up re-analysis in the future. Some of the sample is set aside (8×5 mL at -80°C) while most of the remainder (1.8 L) in step 1 is subjected to solid phase extraction with a “porous extraction paddle (PEP)” containing a mixture of sorbent particles as described^[1]. In steps 2–4 an aliquot of the dried, urine-exposed sorbent particles is eluted and the eluate is subjected to extraction on a polymeric weak anion exchanger. In the second stage of the method (steps 5 and 6), analysis is done by UHPLC-UV with droplet collection as spots onto a MALDI plate followed by MALDI-TOF-MS and MALDI-TOF/TOF-MS. More detail on these steps is provided below.

Analytical scheme: details

In step 1 of Figure 1, a relatively small amount (relative to the amount of chemicals in 1.8 L of urine) of nonpolar and nonpolar/polar solid phase extraction particles (2.0 g) is stirred in urine while enclosed in a rigidified porous nylon bag with the intent that the compounds with the greatest degree of nonpolar structure in the urine will be adsorbed preferentially. This strategy is motivated by the assumption that the most nonpolar xenobiotics will tend to be the most bioactive. In step 2, an aliquot (30 mg) of the sorbent particles (2.0 g) from the PEP is extracted dispersively first with 30% acetonitrile, and then with 80% acetonitrile, where each of these eluent solutions is an aqueous solution containing triethylammonium acetate. In this procedure each dispersive elution step is conducted for 30 minutes, and the two eluents applied in step 2 are each capable of disrupting all kinds of noncovalent interactions. Thus, this elution strategy is intended to totally elute of the particles, and thereby enhance reproducibility. Whether or not total elution is truly achieved remains to be studied in more detail in the future. Nevertheless, subjecting the aliquot of the eluted PEP particles to a second round of the eluent conditions of step 2 yielded an eluate with <1% of the UV absorption and mass spectral signals of the sample from the first round of elution. Each eluate is visibly yellow, and essentially no yellow remains (visibly) on the particles after step 1. The two eluates are then combined.

In step 3 of Figure 1, centrifugation is done to remove any residual sorbent particles, and the supernatant is diluted with water to enhance retention of the intended analytes (nonpolar sulfate conjugates) on the polymeric weak anion exchanger of subsequent step 4. To enhance specificity for isolating nonpolar conjugates in this step, we arbitrarily washed the column with 20 bed volumes of 25 mM ammonium acetate followed by 20 bed volumes of 100% methanol prior to slow elution (50 minutes) with 10 bed volumes of 50% aqueous methanol containing 5% ammonium hydroxide (to neutralize the weak anion exchanger). As above, these conditions were selected to enhance recovery and reproducibility.

In step 5 of Figure 1, where a UHPLC separation is done, an on-line trap column was employed and washed thoroughly to not only enrich the intended analytes, but to discard as much background material as possible so that the subsequent capillary UHPLC column was not overloaded. The injection of 5 μ L of the sample at this point into the UHPLC was not arbitrary: resolution in the UHPLC column suffered from a slightly higher injection volume, and a lower injection volume, of course, reduced the height of the mass spectral signals. Thorough washing (4 minutes at 20 μ L/min) of the trap column (5 mm in length) was done prior to its elution into the analytical column. If 18 μ L instead was injected into a 15 mm trap column, the resolution on the analytical column was much lower, so this condition was abandoned.

The UHPLC-UV (260 nm) chromatograms of the six urine samples subjected to steps 1–5 of Figure 1 are shown in Figure 2. Obviously most of the compounds present in these samples are unresolved in this analysis. (Corresponding profiles at 214 and 320 nm are shown in Supplemental Figures S1 and S2.) It is encouraging, for our purposes, that the profile for each urine is unique, while the overall intensity of each profile is similar. Triplicate injection of one of the samples gave the data shown in Figure 3, showing that the UHPLC separation was highly reproducible. Further study of the reproducibility of our method is described later.

In step 6 of Figure 1, altogether 1320 negative ion MALDI-TOF-MS spectra (MS1 mode) were obtained by scanning each MALDI plate (having 220 sample spots) from each of the six urine samples, as indicated in Figure 4. A maximum of 100 of the most intense peaks ($S/N > 20$ after rejection of MALDI matrix ions) was selected from each MALDI spot. Since the analyte peaks were usually spread over 2–3 spots, peaks appearing in adjacent spots were only recorded once. The final precursor ion list for each urine sample contained about 1,800 ions, many of which were repeats (present in more than one urine sample), as given in Figure 4.

Nonpolar sulfates

Our method focuses on the detection of nonpolar urine sulfates in three ways. First, the solid phase extraction particles include nonpolar-based anion exchange particles that are partly nonpolar. Second, this extraction step is conducted in a nonpolar competitive mode (capacity of the PEP is insufficient for the large volume of urine). Third, the weak anion exchanger (step 3 of Figure 1) is washed with 20 bed volumes each of buffer and methanol prior to elution with aqueous methanolic ammonia.

As indicated in Figure 4, and also in step 6 in Figure 1, putative sulfate conjugates were detected in two ways by MALDI-TOF/TOF-MS (MS2 mode) relying on Peak Explorer software: (1) observation of a significant ($S/N > 4$) product ion at $(M-1)-80$ (due to loss of SO_3 from the precursor anion, $M-1$), and/or (2) formation of a significant product ion at m/z 97 (HSO_4^-). These product ions (taking into account their relative intensity) typically are characteristic, respectively, of aryl and alkyl sulfates as noted before).^[12] The resolution of our instrument enables us to discriminate isobaric HSO_4^- and $H_2PO_4^-$; also phosphate species tend to give a pair of peaks at m/z 79 and 97, with m/z 79 dominating, that is not seen for alkyl sulfates. While an ion at m/z 80 was also commonly observed, and used by others to detect sulfates,^[6] we did not utilize it since its intensity usually was low, and it was redundant relative to the ions at $M-1-80$ and m/z 97. In this way, we detected 1129 unique precursor ions that apparently come from sulfate conjugates in the overall set of six urine samples tested, as indicated in Figure 4. The $M-1$ masses of 1129 unique precursor ions from six urine samples are listed in Supplemental Table S1, and range from m/z 257.010 to 840.243. Assuming that all of these ions truly arise from sulfates, the number of nonpolar sulfates actually present no doubt is larger than this because of isomers.

It is interesting that we have detected more nonpolar sulfates in urine (1129) than Lafaye et al.^[6] (about 15), where both procedures involve reversed-phase LC/MS/MS, and rely on the same fragmentation properties of the precursor negative ions. Probably some combination of the following differences in the two methods, aside from the different sources of urine samples, provides an explanation: (1) we initially concentrated nonpolar sulfates by competitive solid phase extraction using a mixed bed including a nonpolar strong anion exchanger, in contrast to their extraction of urine with an HLB cartridge; (2) only we included (in a second extraction step) a weak anion exchange extraction step; (3) the analytical columns and mobile phase conditions were different; (4) we employed MALDI while they used ESI, which might differ in sensitivity for nonpolar sulfates (indeed, they employed three modes of ESI in order to deal with the problem of analyte-dependent response); and (5) only our mass spectrometer provided high resolution. Additional studies would be required to sort this out, and it will be interesting to subject our samples to LC/ESI-MS/MS in the future, especially because this technique tends to be more convenient than LC/MALDI-MS, although sometimes with a greater susceptibility to ion suppression and enhancement effects, and more restrictions on mobile phase conditions.

The variation in the sulfateome in terms of number of sulfates for the six urine samples is summarized in Table 1. As seen, the total number of putative sulfates (aside from isomers) in each urine varies from 550 to 711, a 1.3-fold range. When either the $M-1-80$ or m/z 97 ion was quite intense, then both of these ions could be seen in the spectrum. We removed the duplicates in these two lists before we calculated the total sulfates for each sample. This explains why the number of total sulfates for each urine is less than the sum of $M-1-80$ and m/z 97 sulfates.

Nomenclature

The experimental mass spectral data in METLIN mostly comes from detection of protonated and deprotonated molecules. To help in clarifying our further discussion here, we will refer

to this published data as $M'+1$ and $M'-1$, respectively, while we will continue to designate the precursor ions that we have detected as $M-1$.

Sulfate hits in METLIN

Workers tend to detect sulfates in a negative ion mode when using a mass spectrometer, and, if they add these compounds to METLIN, they load them in as negative ions. Accordingly, we searched METLIN for $M'-1$ ions that matched (± 20 ppm) the $M-1$ ions in our 1129 peak list (Supplemental Table S1). This gave 3406 hits as noted in Figure 4. Manual examination of this list of anions for sulfate gave 150 hits as pointed out in Figure 4. The 150 compounds are listed in Supplemental Table S2, and discussed in more detail later.

Glucuronides

Our method is less inclined to detect glucuronides than sulfates for two reasons. First, glucuronides in general should be less retained than sulfates on the weak anion exchanger used in sample preparation under the washing conditions we employed. Second, sulfates in general are expected to give stronger signals by negative ion MALDI-TOF-MS than glucuronides (and this is our impression even though we have not tested this carefully). Nevertheless, we did detect some glucuronides by monitoring $M-1-176$ in MALDI-TOF/TOF-MS, corresponding to loss of a glucuronide moiety as a neutral. The number of nonsulfated glucuronides that we detected ranged from 49 to 105 in each of the six urines as summarized in Table 1. Some of the sulfates that we detected (as discussed above) also contained a glucuronide group. These double conjugates, the number of which is listed as well in Table 1, ranged in number from 21 to 51 over the 6 urine samples. The double conjugates in this Table are also part of the list of sulfates.

Xenobiotics hits in METLIN

Most of the ions in METLIN come from measurements of $M'+1$. We are observing $M-1$ from putative sulfates. To obtain hits from contaminants listed as $M'+1$ in METLIN, we adopted the following strategy. First, we searched the $M'+1$ ions in METLIN for $M-1-93.937$, corresponding to $M-1-OSO_3+2H$, since nonpolar compounds, at least in part, tend to undergo oxidation to an alcohol followed by sulfation *in vivo*. This gave 6156 hits as indicated in Figure 4. We then matched this list by keyword using MATLAB with lists of 334 pesticides,^[13] 426 herbicides,^[14] and 129 priority pollutants^[15] that we refer to as “xenobiotics”. This led to 35 hits, as presented in Table 2.

We similarly searched the $M'+1$ compounds in METLIN for the above contaminants based on the assumption that such compounds would be detected in our method after undergoing oxidation to an alcohol followed by glucuronidation *in vivo*. Thus, we searched $M'+1$ compounds in METLIN having masses $M-1-190$ (corresponding to $M-1-C_6H_8O_6-O+2H$), against the compounds in the above contaminant lists using MATLAB. However, this gave no hits, apparently because our method, for the reasons given above, did not have enough sensitivity to detect contaminant glucuronides in these samples.

The intended meaning for us of the list of xenobiotics provided in Table 2 is only to begin to suggest compounds for subsequent quantitative targeted analysis (including use of internal

standards) of urines from cases vs controls for a health or disease condition of interest. Before any targeted analysis is undertaken, the ions suggesting the presence of these compounds need to be identified with much higher certainty than is done here. This would require measurement at a higher mass spectral resolution and/or fragmentation analysis, combined finally with testing of authentic compounds to confirm assignments. Also, unless a way can be found to correlate exposure to a given contaminant with the amount of its sulfate found in urine, then such targeted analysis in the future needs to be based on a method in which the total amount of the compound present in urine is measured, as by deconjugating the urine at the outset of the analytical procedure, a common technique.^[16] The only compounds in Table 2 that are the same type as these reported previously to correlate with preterm birth^[2–5] are the two phthalates.

In spite of the multiple factors that can influence the amount of a xenobiotic as sulfate in urine after a given exposure, we speculate that it might be possible for a correlation to emerge between the exposure to a contaminant and the level of its sulfate in urine when a relatively large number of samples is tested. This would require the measured urine levels of the sulfates to be normalized. One normalization strategy to consider is dividing the level of a given sulfate by the total intensity of the sulfates in that urine which are shared in common among all samples. This would be analogous to the strategy to normalize a given metabolite relative to a person's shared metabolome.^[17] As seen in Supplemental Table S3, the intensity of the overall sulfateome varied by a factor of 2.6 in the urines that we tested for loss of 80; by 3.7 for m/z 97; and by 2.7 for the sum of these ions.

Nonpollutant hits in METLIN

Endogenous compounds, of course, also are of interest as biomarkers for a health conditions or disease of interest. Accordingly, we evaluated the putative sulfates that we detected by MS for two classes of biomolecules that are expected to contribute significantly to the urine nonpolar sulfateome: steroids and flavonoids. We also similarly evaluated the glucuronides that we detected, as described below.

Steroids

We searched for steroids hits in METLIN in two ways. First, we manually examined the sulfates pointed out in Figure 4, and listed in Supplemental Table S2, for steroids. This gave 27 steroid hits as also noted in Figure 4. Second we assumed that METLIN also contained steroids as nonsulfates in its collection of compounds detected as $M' + 1$, and that many of these steroids are alcohols. This latter assumption is based on the fact that the 20 common human steroids (Supplemental Table S4) are either alcohols, or give a steroid which already is one of the 20 common human steroids (or an isomer thereof) by metabolic hydroxylation. Under the further assumption that such steroids would give an $M' - 1$ ion in our method after undergoing sulfation *in vivo*, we thereby searched the $M' + 1$ compounds in METLIN for masses $M - 1 - 77.941$, corresponding to $M - 1 - \text{SO}_3 + 2\text{H}$. This gave a list of 6727 compounds as pointed out in Figure 4. In turn, using MATLAB, we searched this list for the names of 20 common steroids. This gave 122 steroid hits, as indicated in Figure 4. The names of the compounds are listed in Supplemental Table S5.

Flavonoids

Comparing the 150 sulfate hits of Figure 4 by compound name using MATLAB with a list of 5158 flavonoids^[18] gave 59 flavonoid sulfate hits (Figure 4), which are listed in Supplemental Table S6. We also searched the above 6727 hits by compound name using MATLAB against a list of 5158 flavonoids, which gave 1582 flavonoid hits as indicated in Figure 4 and listed in Supplemental Table S7.

Examples of sulfate and glucuronide conjugates

We postulate that the peak at m/z 364.086 (Figure 5a) is from morphine sulfate (exact mass 364.085), since the agreement is 3 ppm. This peak was found in all of the samples, with a 5-fold range in peak height (data not shown). As seen in Figure 5b, this compound in an automated MS2 batch run formed both (M-1)-80 (m/z 284) and SO3 (m/z 80) product ions, where the former product ion is favored since it arises from a cleavage yielding a phenolate moiety. We have assigned the data shown in Figure 6, as seen, to 3 β , 16 α -dihydroxyandrostene sulfate (DHEAS, accurate mass 383.151 observed in Figure 6a, exact mass 383.153, 5 ppm). As seen in Figure 6b, from an MS2 analysis, the peak at m/z (HSO₄⁻) dominates, while m/z 303 (alkoxylate) from loss of SO₃ is small, consistent with the compound being an alkyl sulfate. A moderate peak also is seen at m/z 80 (SO₃). This compound is one of the steroids elevated in pregnancy. In Figure 7 is shown data that may arise, as indicated, from estradiol 3-sulfate 16-glucuronide, a double conjugate. The isomer, β -estradiol 3-(β -D-glucuronide) 17-sulfate, is another possibility. The mass measured in Figure 7a (543.151) is within 5 ppm of the exact mass (543.154), and the product ions observed in Figure 7b, in an MS2 experiment, fit losses of a glucuronide group and SO₃, and formation of SO₃⁻.

Reproducibility

High reproducibility is of paramount interest for us because we also seek to discover biomarkers for preterm birth by comparing the mass spectral features in an unsupervised way of biosamples from women who have experienced a preterm vs term birth. We optimized the ruggedness and reproducibility of our method in several ways as follows. We selected conditions (e.g. dispersive extraction; slow, large volume elution) that should take each step to completion. Precipitates and evaporations were minimized. Initially we evaporated the supernatant of step 2 prior to step 3 of Figure 1, but redissolving it gave a cloudy solution, so instead we diluted it prior to this latter step. We injected relatively clean samples into the UHPLC system, and avoided overloading of the column. MALDI-TOF-MS data (beyond the injection of a sample into the UHPLC in triplicate as described above) were collected with extensive, automated signal averaging from each spot.

To evaluate the reproducibility of the method in a preliminary way (but beyond the initial test of reproducibility shown in Figure 3), we repeated the entire procedure six weeks later, starting with new aliquots of the urine-exposed solid phase extraction particles (stored at -80 °C). Representative UHPLC-UV data from one of the samples is shown in Figure 8. It is encouraging that the corresponding chromatographic peak patterns in the two sets of data are found to highly overlap even though the data came from different runs six weeks apart.

We also evaluated the reproducibility of the retention times (in terms of spot numbers on the MALDI target from the UHPLC separation, where the collection rate was three spots per minute) for representative analytes: several steroid sulfates were selected for this purpose. Without normalization, when a given spot n (n ranges from 1 to 220 over the 80 min UHPLC elution, 3 drops/min) from one urine sample had the highest intensity for a given steroid, then another urine sample, at most, had a corresponding spot at $n \pm 3$ or less. Once the retention data was normalized by a simple linear regression method, using three distinctive m/z to generate the alignment parameters, the precision was $n \pm 1$. Thus, our method is highly reproducible. Nevertheless, in practice it will be best to analyze a set of samples to be compared as a single batch.

CONCLUSION

We have developed a qualitative method comprising solid phase extraction, UHPLC, and mass spectrometry steps for the human urine nonpolar sulfateome that is interesting in three ways. (1) For a single urine, we have expanded the measurement of the urine sulfateome by about 35–50 fold, and, for a collection of 6 samples, about 75 fold. (2) The method provides a strategy to discover the masses of nonpolar xenobiotics to which humans are exposed, helping to design a subsequent study utilizing quantitative targeted analysis: the method provides a pathway to some of the nonpolar xenobiotic exposome. Steps and conditions were developed for sample preparation that achieved high reproducibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grant P42WS017198 from NIEHS.

References

1. Shao G, MacNeil M, Yao Y, Giese RW. Porous Extraction Paddle: A solid phase extraction technique for studying the urine metabolome. *Rapid Commun Mass Spectrom*.
2. Cantonwine D, Meeker JD, Hu H, Sanchez BN, Lamadrid-Figueroa H, Mercado-Garcia A, Fortenberry GZ, Calafat AM, Tellez-Rojo MM. Bisphenol an exposure in Mexico City and risk of prematurity: a pilot nested case control study. *Environ Health Perspectives*. 2010; 9:62.doi: 10.1186/1476-069X-9-62
3. Meeker JD, Hu H, Cantonwine DE, Lamadrid-Figueroa H, Calafat AM, Ettinger AS, Hernandez-Aliva M, Loch-Carusio R, Tellez-Rojo MM. Urinary Phthalate Metabolites in Relation to Preterm Birth in Mexico City. *Environ Health Perspectives*. 2009; 117:1587.doi: 10.1289/ehp.0800522
4. Pelka-Mucha A, Venners SA, Sjodin A, Jones R, Anderson S, Yu Y, Wang X. Association Between PBDE Exposure and Preterm Birth. *BFR Program*. 2008:39. Abstract No. 23.
5. Tsukimori K, Tokunaga S, Shibata S, Uchi H, Nakayama D, Ishimaru T, Nakano H, Wake N, Yoshimura T, Furue M. Long-Term Effects of Polychlorinated Biphenyls and Dioxins on Pregnancy Outcomes in Women Affected by the Yusho Incident. *Environ Health Perspectives*. 2008; 116:626.
6. Lafaye A, Junot C, Ramounet-Le Gall B, Fritsch P, Ezan E, Tabet J-C. Profiling of sulfoconjugates in urine by using precursor ion and neutral loss scans in tandem mass spectrometry. Application to the investigations of heavy metal toxicity in rats. *J Mass Spectrom*. 2004; 39:655.doi: 10.1002/jms.635 [PubMed: 15236304]

7. Zhang T, Creek DJ, Barrett MP, Blackburn G, Watson DG. Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with Orbitrap mass spectrometry for metabolomic studies of human urine. *Anal Chem.* 2012; 84:1994.doi: 10.1021/ac2030738 [PubMed: 22409530]
8. Roux A, Xu Y, Heilier J-F, Olivier M-F, Ezan E, Tabet J-C, Junot C. Annotation of the human adult urinary metabolome and metabolite identification using ultra high performance liquid chromatography coupled to a linear quadrupole ion trap-Orbitrap mass spectrometer. *Anal Chem.* 2012; 84:6429.doi: 10.1021/ac300829f [PubMed: 22770225]
9. Strahm E, Kohler I, Martel SS, Carrupt P-A, Veuthey J-L, Saugy M, Saudan C. Isolation and quantification by high-performance liquid chromatography-ion-trap mass spectrometry of androgen sulfoconjugates in human urine. *J Chromatogr A.* 2008; 1196:153.doi: 10.1016/j.chroma.2008.04.066 [PubMed: 18501914]
10. Balcells G, Pozo OJ, Esquivel A, Kotronoulas A, Joglar J, Segura J, Ventura R. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr A.* 2005; 389:65.doi: 10.1016/j.chroma.2015.02.022
11. van der Hooft JJ, de Vos RCH, Mihaleva V, Bino RJ, Ridder L, de Roo N, Jacobs DM, Van Duynhoven JPM, Vervoort J. Structural Elucidation and Quantification of Phenolic Conjugates Present in Human Urine after Tea Intake. *Anal Chem.* 2012; 84:7263. [dx.doi.org/10.1021/ac30173391](https://doi.org/10.1021/ac30173391). [PubMed: 22827565]
12. Zhang H, Henion J. Quantitative and Qualitative Determination of Estrogen Sulfates in Human Urine by Liquid Chromatography/Tandem Mass Spectrometry Using 96-Well Technology. *Anal Chem.* 1999; 71:3955. [PubMed: 10500482]
13. Pesticide list. <http://www.ars.usda.gov/services/docs.htm?docid=14147>
14. Herbicide list. <http://wssa.net/weed/herbicides/>
15. Priority pollutant list. <http://water.epa.gov/scitech/methods/cwa/pollutants.cfm>
16. Roca M, Leon N, Pastor A, Yusa V. Comprehensive analytical strategy for biomonitoring of pesticides in urine by liquid chromatography-orbitrap high resolution mass spectrometry. *J Chromatogr A.* 2014; 1374:66. [org/10.1016/j.chroma.2014.11.010](https://doi.org/10.1016/j.chroma.2014.11.010). [PubMed: 25499061]
17. Warrack BM, Hnatyshyn S, Ott KH, Reily MD, Sanders M, Zhang H, Drexler DM. Normalization strategies for metabolomics analysis of urine samples. *J Chromatogr B.* 2009; 877:547.doi: 10.1016/j.jchromb.2009.01.007
18. Flavonoid list. <http://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=ProcessClassSearch&LMID=LMFA08>

Figure 1.

Scheme for analysis of the nonpolar urine sulfateome. PEP: porous extraction paddle (a rigidified “teabag” containing a mixture of solid phase extraction particles that is stirred in the urine).

Figure 2. UHPLC-UV (260 nm) chromatograms of six urine samples subjected to steps 1–5 of Figure 1.

Figure 3.
UHPLC-UV chromatograms from three successive injections of the sample (step 5 of Figure 1 from urine E).

Figure 4. Detection of precursor ions (as $M-1$) nonpolar sulfates in urine by MS1 and MS2 experiments, along with results of searching candidate compounds (designated as “hits”).

Figure 5.

Apparent detection of morphine-3-sulfate by the method described in Figure 1. **a**, Detection of the precursor ion as M-1 at 364.086 m/z (3 ppm), by MALDI-TOF-MS. **b**, Detection and assignment of the two product ions of putative morphine-3-sulfate (m/z 80 and 284) by MALDI-TOF/TOF-MS.

Figure 6.

Apparent detection of 3β,16α-dihydroxyandrost-4-en-20-one sulfate by the method described in Figure 1. **a.** Detection of the precursor ion as M-1 at 383.151 m/z (5 ppm) by MALDI-TOF-MS. **b.** Detection and assignment of three product ions (m/z 80, 97 and 303).

Figure 7.

Apparent detection of estriol-3-sulfate-16-glucuronide by the method described in Figure 1.

a. detection of the precursor ion as M-1 at 543.151 m/z by MALDI-TOF-MS (5 ppm). **b.** Detection and assignment of three product ions (m/z 80, 463 and 367) by MALDI-TOF/TOF-MS.

Figure 8.

Analysis of urine E by steps 1-5 of the method shown in Figure 1 at time = 0 (curve 1) and time = 6 weeks (curve 2).

Variation in the number of nonpolar putative sulfates (and the number of glucuronides) in the six urines by UHPLC/MALDI-TOF/TOF-MS

Table 1

	Number of Sulfates		Total	Glucuronides		Double Conjugates
	M-1-80	m/z 97		M-1-176		
Urine						
A	424	325	629	94		47
B	275	353	550	49		21
C	350	324	549	105		51
D	392	286	576	57		30
E	398	451	711	81		43
F	332	475	691	86		43

Table 2

Pesticide, herbicide and priority contaminant hits in METLIN based on detection of the 1129 precursor ions of Figure 4 and assuming that the compounds underwent oxidation to an alcohol followed by sulfation *in vivo*. Note that several compounds are listed as both herbicides and pesticides.

Herbicide	Pesticide	Priority pollutant
benzofenap	benomyl	di-n-octyl phthalate
butralin	cinmethylin	diethyl phthalate
cinmethylin	dinitramine	indeno (1,2,3-cd) pyrene
clethodim	dinoseb	
difenoxuron	ethofumesate	
dinitramine	flumetralin	
dinoseb	imazethapyr	
dinoterb	isopropalin	
ethofumesate	methiocarb	
imazethapyr	metolachlor	
isopropalin	myclobutanil	
mefenacet	napropamide	
metolachlor	oxadiazon	
napropamide	pirimiphos ethyl	
oxadiazon	sethoxydim	
phenobenzuron	thiobencarb	
prosulfocarb	naphthalene ¹	
quizalofop	quizalofop ²	
sethoxydim	rotenone ³	
thiazopyr		
thiobencarb		
tralkoxydim		

The derivatives of pesticides with superscripts 1, 2 and 3 were found using our method.

¹ 1-Nitro-5,6-dihydroxy-dihydronaphthalene

² Ethyl quizalofop

³ Dihydrorotenone