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Quantification of Twenty-one Metabolites of Methylnaphthalenes and Polycyclic Aromatic Hydrocarbons in Human Urine

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) and their alkylated derivatives, such as methylnaphthalenes (MeNs), are harmful pollutants ubiquitously present in the environment. Exposure to PAHs has been linked to a variety of adverse health effects and outcomes, including cancer. Alkyl PAHs have been proposed as petrogenic source indicators because of their relatively high abundance in unburned petroleum products. We report a method to quantify 11 urinary methyl naphthols (Me-OHNs), metabolites of 1- and 2-methylnaphthalenes, and 10 monohydroxy PAH metabolites (OH-PAHs), using automated liquid-liquid extraction and isotope dilution gas chromatography tandem mass spectrometry (GC-MS/MS). After spiking urine (1 mL) with ^{13}C-labeled internal standards, the conjugated target analytes were hydrolyzed enzymatically in the presence of ascorbic acid. Then, their free species were preconcentrated into 20% toluene in pentane, derivatized and quantified by GC-MS/MS. The 11 Me-OHNs eluted as 6 distinct chromatographic peaks, each representing 1–3 isomers. Method detection limits were 1.0–41 pg/mL and the coefficients of variance in quality control materials were 4.7–19%. The method was used to analyze two National Institute of Standards and Technology's Standard Reference Materials and samples from 30 smokers and 30 non-smokers. Geometric mean concentrations were on average 37 (Me-OHNs) and 9.0 (OH-PAHs) fold higher in smokers than in non-smokers. These findings support the usefulness of Me-OHNs as potential biomarkers of non-occupational exposure to MeNs and sources containing MeNs.

Keywords

Polycyclic aromatic hydrocarbon; PAH; methylnaphthalene; human exposure; biomonitoring; biomarker

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) and their alkylated derivatives, such as methylnaphthalenes (MeNs), are ubiquitous environmental pollutants [1–3] that come from two types of sources. Pyrogenic PAHs are formed through the incomplete combustion of organic materials, such as fossil fuel and biomass. At high combustion temperature, pyrogenic sources emit mainly un-substituted PAHs, while at lower temperature below approximately 700ºC – such as in tobacco smoking – a larger amount of alkyl PAHs, mainly methyl derivatives, are generated [4–6]. Petrogenic sources refer to unburned petroleum products, such as oil spills, leaks and road oil drip evaporation, and contain more alkylated PAHs than do pyrogenic sources [7–9]. The concentration ratio of alkyl PAHs to their un-substituted homologues has been used as an indicator of unburned fossil fuel sources in the atmosphere, soil, and aquatic environments [7, 10–14].

MeNs and naphthalene are among the most abundant PAH congeners in polluted air such as urban, roadside and airport air [15–17], and biomass smoke [18], often at concentrations that far exceed those of less volatile PAHs, such as benzo[*a*]pyrene (BaP). Furthermore, MeNs are industrial chemicals used to make dyes, resins, and many other consumer products [19]. In 2010, 28 million cereal boxes were recalled in the United States after 2-MeN was suspected of leaching out of the packaging [20, 21]. This incident led to a public calling for additional research on MeNs, particularly to better understand their potential health effects.

Some PAHs and chemical mixtures containing PAHs, such as BaP, coal tar pitch, coke production, and chimney sweep soot, are classified as Group 1 human carcinogens by the International Agency for Research on Cancer [22]. Many other PAHs, including naphthalene, are probable or possible human carcinogens [23]. A recent study reported a dose-response relationship between urinary naphthalene metabolites and chromosomal aberrations – established markers of cancer risk – in school-age children [24]. In addition, PAHs have been linked to a variety of adverse health effects and outcomes [3]. While toxicities and health effects associated with exposure to un-substituted PAHs have been widely studied, such information is limited on MeNs [19, 25], even though the Agency for Toxic Substances & Disease Registry includes MeNs on its list of toxic substances [19]. A few animal studies on mice and in-vivo essays have indicated dose- and time-dependent lung toxicity after acute exposure to MeNs [26–32]; the severity of lung toxicity associated from 2-MeN exposure was similar to that from naphthalene and worse than that from 1-MeN [27, 28, 33].

In children and the general population from various countries [34–38], and populations with high occupational exposure to PAHs [39, 40], exposure has been assessed by measuring their monohydroxlated metabolites (OH-PAHs) in urine, with 1-hydroxypyrene (1-PYR) being the most commonly used exposure biomarker [41–45]. To the best of our knowledge, no studies have reported measurements of MeNs metabolites in human samples.

We report the development and validation of a method to quantify 11 metabolites of 1-MeN (n=5) and 2-MeN (n=6), as well as 10 metabolites of 4 un-substituted PAHs, i.e. naphthalene (n=2), fluorene (n=3), phenanthrene (n=4), and pyrene (n=1), in human urine.

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The method used enzymatic deconjugation, automated liquid-liquid extraction (LLE), and isotope dilution gas chromatography tandem mass spectrometry (GC-MS/MS). The method was then used to quantify PAH and MeN metabolites in urine specimens from smokers and non-smokers.

Methods and materials

Standards, chemicals and supplies

All reagents and solvents used were of the highest available grade or intended for pesticide residue analysis. Pentane and toluene (>99.8%) were obtained from Tedia Company Inc. (Fairfield, OH, USA). Sodium acetate anhydrous (>99.0%) was purchased from Fisher Scientific (Pittsburg, PA, USA), dodecane (99%), ascorbic acid, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), and β-glucuronidase type H-1 with sulfatase activity (β-glucuronidase 300,000 units/g, sulfatase 10,000 units/g), isolated from *Helix pomatia,* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-high purity nitrogen and helium were obtained from Airgas South Co. (Chamblee, GA, USA). $^{13}C_{12}$-2,3,3′,4,4′-pentachlorobiphenyl (^{13}C-PCB105) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All native and ^{13}C-labeled OH-PAH and Me-OHN analytes, their abbreviations, together with their suppliers are listed in Table 1.

Stock solutions of individual OH-PAHs prepared from neat materials (ca. 1 mg/mL in acetonitrile) and purchased individual Me-OHN stock solutions (50 μg/mL in isooctane) were used to prepare standard mixtures for eight calibration standards at concentrations ranging from 1–1,000 pg/μL, except for 1- and 2-hydroxynaphthalene (1-NAP and 2-NAP) which were present at four times higher concentrations (4–4,000 pg/μL). The ^{13}C-labeled spiking solution contained eleven ^{13}C-labeled internal standards (^{13}C-IS), i.e. 10 labeled OH-PAHs and $^{13}C_6$-2-methyl-1-naphthol in acetonitrile (100 pg/μL for ^{13}C-labeled 1- and 2-NAP, 25 pg/μL for the remaining ^{13}C-IS). The recovery standard solution contained ^{13}C-PCB105 (200 pg/μL in toluene).

Human urine samples

Two Standard Reference Materials (SRMs), SRM 3672 (Smoker urine) and SRM 3673 (Non-smoker urine), were obtained from the U.S. National Institute of Standards and Technology (NIST). Thirty urine specimens collected from 30 self-identified smokers were purchased from Bioreclamation, LLC (NY, USA). Thirty non-smoker urine specimens were collected anonymously at the Centers for Disease Control and Prevention (CDC) through a convenience sampling of adult volunteers. All urine specimens were refrigerated upon collection, and stored at −70°C until use. CDC's Human Subjects Institutional Review Board approved the anonymous collection of urine for method development and validation. A waiver of informed consent was requested under 45 CFR46.116(d).

Sample preparation procedure

Urine (1 mL) was fortified with ^{13}C- IS mixture (40 μL) using a Gilson 215 liquid handler (Gilson Inc., Middleton, WI, USA). The weight of the vial containing the ^{13}C- IS mixture was recorded before and after spiking of the sample run to calculate spiking accuracy for the

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run. Then, the urine was spiked with sodium acetate buffer (pH 5.5, 1 M, 1 mL) containing β-glucuronidase/sulfatase (3000 unit β-glucuronidase and 100 unit sulfatase activity/mL buffer) and ascorbic acid solution (10 μL, 250 mg/mL). After overnight incubation at 37°C (~18 hours), the target analytes were extracted with a 20% toluene 80% pentane solvent mix (v/v) through automated LLE on a Gilson 215 liquid handler as described elsewhere [46]. In brief, de-ionized water (2 mL) and pentane/toluene mix (5 mL) were added to the urine and the samples were mixed by rotation for 5 min (20 rpm) using the automatic liquid handler. The procedure was paused and the samples were centrifuged for 20 min (2800 rpm). After the samples were returned to the liquid handler, the organic phase was transferred to collection tubes. This extraction procedure was repeated with additional pentane/toluene mix volume (5 mL). The combined pentane/toluene extracts were spiked with dodecane (10 μL) as a keeper, and concentrated on a RapidVap vacuum evaporator (Labconco Corporation, Kansas City, MO, USA), first at 40°C, 40% vortex speed and 500 mbar vacuum for 10 min to evaporate pentane, then at 70°C, 50% speed and 230 mbar to evaporate toluene until ~10 μL remained (20 min). The concentrated extracts were reconstituted with toluene (20 μL), spiked with recovery standard solution ^{13}C-PCB105 (10 μL), and transferred to GC vials with 300-μL inserts. The target analytes were derivatized to their trimethylsilyl derivatives by adding MSTFA (10 μL) and incubating at 60° for 30 min.

Isotope dilution GC-MS/MS

GC-MS/MS analysis was carried out on an Agilent 7000B triple quadrupole mass spectrometer, interfaced with a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), operating in the multiple reaction monitoring (MRM) mode using electron impact ionization. One microliter of the extract was injected in splitless mode using a liner packed with glass wool to minimize column contamination at an inlet temperature of 270ºC. The chromatographic separation was carried out on a Zebron ZB-5MS column (30 m × 0.25 mm, 0.25 μm film thickness, Phenomenex Corp, Torrance, CA), under a constant flow of 1 mL/min helium. The initial oven temperature was 95ºC (1 min), then ramped at 15ºC/min to 195ºC, 2ºC/min to 205ºC and held for 3 min, and finally ramped at 40ºC/min to 320ºC and held for 3 min (total run time 22 min/sample). The transfer line and source temperatures were 270 ºC. In the triple quadrupole collision cell, helium was used as the quench gas at 2.25 mL/min and nitrogen was the collision gas at 1.5 mL/min. The instrument sensitivity was checked daily by injecting the lowest calibration standard (1 pg injected on column for 1-NAP and 2-NAP and 250 fg for the remaining analyte). For the daily instrument sensitivity check to pass, the signal-to-noise (S/N) ratio needed to be at a minimum of 10 for all analytes.

Table 1 lists the GC-MS/MS parameters, including precursor and product ions, collision energy and retention time, for all target analytes. Each of the 10 OH-PAH analytes is quantified with its corresponding ^{13}C-IS. All Me-OHN metabolites were quantified with $^{13}C_6$-2-methyl-1-naphthol (2M1N) as the internal standard. Identity of the Me-OHN peaks in urine samples was confirmed by the use of mass peak profiling [47] on a magnetic sector high-resolution mass spectrometer (MAT95XL, Thermo Fisher Scientific Inc. Waltham, MA, USA) at mass resolution of 10,000 (Supplementary Material, Accurate mass peak profiling for peak confirmation).

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Quality assurance and quality control

Anonymously collected urine was pooled, pressure-filtered through a 0.45–μm SuporCap–100 Capsule (Pall Corp. Ann Arbor, MI, USA), diluted with de-ionized water (1 part water to 4 parts of filtered urine), and used as a starting pool to make quality control (QC) materials. This pool was then divided and spiked with a standard mixture containing the target analytes in acetonitrile at two different levels to create two QC pools: low QC (spiked with 200 pg/mL) and high QC (spiked with 700 pg/mL). The spiked urine pools were stirred at room temperature overnight, after which the QC materials were aliquoted into 16 ×100 mm glass culture tubes, and stored at −70°C until use. Each QC pool was characterized by producing 30 analytical runs over a period of three months to determine the 95% and 99% control limits. A multi-rule QC system was adopted to monitor within- and between-run variability for each analytical run using replicates of the two QC pools per run [48]. The concentrations of the QCs in each run were evaluated using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

In this method, one analytical run is defined as 34 unknown urine samples, two water blanks, two low QCs, two high QCs and eight calibration standards which are derivatized and analyzed in parallel with the urine extracts. For each sample, analyte results were considered valid when fulfilling the following criteria: (i) relative retention time, defined as the retention time ratio of a native analyte over its ^{13}C-IS, was within ± 0.25% of the reference ratio set by the average of the calibration standards, (ii) calculated recovery of the individual ^{13}C-IS in the sample was within 25–150%, (iii) ^{13}C-IS spiking accuracy for the run, defined as the actual weight of the ^{13}C-IS spiking solution used divided by the theoretical weight of spiking solution required for the run, was within 90–110%, and (iv) the QCs in the run passed the multi-rule QC check as detailed elsewhere [48].

Results and discussion

We developed a method to quantify 11 hydroxylated metabolites of 1-MeN and 2-MeN, along with 10 OH-PAHs, metabolites of 4 un-substituted PAHs, in human urine using automated LLE and isotope dilution GC-MS/MS determination. To the best of our knowledge, this is the first report of an analytical method for measuring MeN metabolites in human urine and first report of their concentrations in urine samples. As expected, the Me-OHNs eluted shortly after the naphthols and before the hydroxyfluorenes (Figure 1). Although analytically this method could measure five additional compounds (1-methyl-2-naphthol, 2-methyl-1-naphthol, 8-methyl-1-naphthol, 9-hydroxyphenanthrene, 3-hydroxyfluoranthene), as shown in Figure 1, these compounds were not reported because of their instability and unstable QC curves, which rendered them inadequate as exposure biomarkers.

GC-MS/MS of Me-OHNs

We evaluated several GC columns with various phase polarities from multiple manufactures for their separation capabilities. On a ZB-5MS 5% phenyl methyl silicone column, the 14 Me-OHNs eluted as 8 distinct chromatographic peaks with each peak representing 1–3 isomers (Figure 1). Columns with intermediate polarity, such as RTX®-440 and RTX®-50

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(Restek, Bellefonte, PA, USA), gave similar separation as the non-polar ZB-5MS (data not shown). RTX®-2330 (Restek, Bellefonte, PA, USA), a polar column with its stationary phase consisting of 90% biscyanopropyl/10% cyanopropylphenyl polysiloxane, yielded the best separation in which the 14 Me-OHNs were eluted as 11 chromatographic peaks (Supplementary Material, Figure S1). However, when using this column, the analytes elution slowed down. To maintain the separation for the rest of the analytes, the GC program run time had to be increased to 40 min. This led to broadened peak shape and reduced sensitivity for the late eluting analytes such as 1-PYR. Therefore, we chose a ZB-5MS column and reported the combined concentration of co-eluting metabolites. Such combined concentrations should provide a better estimation of MeN exposure than individual concentrations because 1-MeN and 2-MeN are both present in pyrogenic and petrogenic sources and both lead to multiple metabolites. However, for certain applications, such as to assess accidental exposure to specific MeNs, the RTX-2330 column would provide a better separation of Me-OHN metabolites than the ZB-5MS column and thus facilitate the exposure assessment to 1-MeN or 2-MeN separately.

The mass spectrometer was at MRM mode. For most analytes, the molecular ion was chosen as the precursor ion, and M-30 (- 2[$\cdot CH_3$]), M-31 (- [$\cdot CH_3 + CH_4$]) or M-89 (-$OSiC_3H_9$) were selected as the product ions (Table 1, Supplementary Material, Figure S2). During the method validation, a partially co-eluting interference affecting 4-hydroxyphenanthrene was present in certain urine samples. This interference was eliminated when using a different precursor-product ion pair, M-31 to M-46 (- [$\cdot CH_3 + \cdot CH_3 + CH_4$]), demonstrating the superior selectivity of MS/MS.

Enzymatic deconjugation

Like the OH-PAH metabolites [46], preliminary data suggest that the Me-OHNs are excreted in urine predominantly as conjugates (data not shown). However, the method can only quantify the de-conjugated metabolites because analytical standards are available for the hydroxyl metabolites, but not the conjugates. Therefore, it is essential to completely convert the conjugated metabolites to their free forms. This method used overnight hydrolysis of urine samples (1 mL) with 10 mg enzyme (3000 unit β-glucuronidase activity and 100 unit sulfatase activity) to reach complete deconjugation of all OH-PAHs [46]. This is consistent with the optimal condition (20,000 unit β-glucuronidase and 16 h hydrolysis for 10 mL artificial urine fortified with 1-PYR glucoronide) for 100% deconjugation reported by Wegener et al. [49]. However, when we conducted deconjugation experiments on the Me-OHNs, concentrations of several Me-OHNs, such as 4-methyl-1-naphthol (4M1N), decreased up to 50% during the overnight hydrolysis (Figure 2). Potential explanations for such decrease include physical losses, such as those resulting from binding of the free species to the culture tube glass surface, or chemical changes, such as oxidation or photodegradation. After excluding glass binding and photodegradation (data not shown), we tested ascorbic acid as an antioxidant to prevent potential degradation by hydroxyl and superoxide radicals [50–52], and found that ascorbic acid effectively stabilized the Me-OHNs during the hydrolysis process by preventing the oxidation of the deconjugated Me-OHNs (Figure 2). We further determined 2.5 mg ascorbic acid per 1 mL urine as the

appropriate amount to ensure that all Me-OHNs were stable during the enzymatic hydrolysis.

Method improvement

In addition to the inclusion of Me-OHNs, the current method improved the quantification of urinary OH-PAHs, including 1-PYR, compared to the previous method [46]. First, the automated ^{13}C-IS spiking improved accuracy and precision (data not shown), and eliminated potential human error in this critical step. The ^{13}C-IS spiking accuracy was calculated and monitored as an additional quality assurance check for an analytical run. Second, the solvent selection choice maximized extraction efficiencies, reduced losses during samples preparation, and therefore, increased method recoveries. The recoveries were 52% and 53% for the two ^{13}C-naphthols and 69–100% for the eight larger ^{13}C-OH-PAH internal standards, compared to 46–72% in the previous method [46]. Third, the method incurred reduced cost and increased productivity by using a considerably cheaper and more robust instrumentation (MS/MS vs. high resolution mass spectrometry). Fourth, the method gave increased sensitivity of the OH-PAH analytes, as demonstrated by higher S/N ratios for the lowest standard (Supplementary Material, Figure S3). Finally, the presence of ascorbic acid facilitated the deconjugation of several OH-PAH conjugates, i.e., those of 1-naphthol and 9-hydroxyfluorene [46]. The hydrolysis of these conjugates was inefficient compared to other OH-PAHs, potentially due to steric hindrance and higher composition of sulfate conjugate [46]. In addition, 9-FLU is an alcohol rather than a phenol, which might affect the deconjugation efficiency. The use of ascorbic acid increased the deconjugation speed, most likely by preventing the oxidative damage of β-glucuronidase and sulfatase [50], and thus reduced deconjugation time required to achieve maximal yield from overnight to 3 hour (Supplementary Material, Figure S4). All of these characteristics together are essential for conducting large epidemiological studies such as the National Health and Nutrition Examination Survey.

Our previous analytical method [46] targeted 24 urinary OH-PAHs, namely the 10 OH-PAHs reported in this method and monohydroxyl derivatives of BaP, chrysene, benz(a)anthracene and benzo(c)phenanthrene, including 3-hydroxybenzo[*a*]pyrene (3-BaP, LOD: 2.6 ng/L), a metabolite of BaP [53]. However, these relatively large PAHs are mainly excreted through feces [3]. Therefore, their monohydroxyl metabolites may not be optimal exposure biomarkers as suggested by the rather infrequent detection of these compounds in the Canadian and US national surveys [36, 38] and in other studies [54, 55]. For example, 3-BaP had a detection rate of 0% in all demographic groups in the Canadian Health Measures Survey (LOD: 2 ng/L) [38]. With a more sensitive method (LOD: 0.1 ng/L) [56], Lafontaine et al. detected 3-BaP in 18.5% of non-smokers and 66.7% of smokers (13–50 cigarettes/day), and acknowledged nonetheless that the use of 3-BaP as PAH exposure biomarker was less justified [57]. To avoid miss-interpretation during exposure assessment, we discontinued the measurement of monohydroxyl derivatives of the larger PAHs, including 3-BaP, and reported 10 detectable and stable urinary OH-PAHs, i.e. metabolites of naphthalene, fluorene, phenanthrene and pyrene, in this method and in all recent research projects [58, 59].

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Method evaluation and validation

The method detection limit (MDL) for this method was defined as 3 times of S_0, where S_0 is the standard deviation (SD) as the concentration approaches zero [60]. Because OH-PAHs and Me-OHNs are ubiquitous contaminants in urine, we spiked synthetic urine with 4 different levels of standard mixtures (10, 20, 50 and 100 pg/mL; 40–400 pg/mL for 1- & 2-NAP) and performed 6 repeated measurements to determine the SD at each level. The SD was then plotted as a function of concentration and S_0 was extrapolated as the intercept of the regression line [60]. The method detection limits were 7.0–41 pg/mL for the Me-OHNs and 1.0–19 pg/mL for the OH-PAHs (Table 2).

The overall coefficients of variance (CVs) from 30 runs of QCL and QCH over a 3-month period, shown in Table 2, were 4.7–8.3% for most OH-PAHs, except for 3-hydroxyphenanthrene (13%). As expected, the between-day variability is higher than the within-day variability. For the Me-OHNs, the CVs were 11–19%, higher than those of OH-PAHs, likely because only one ^{13}C-IS is available for the Me-OHNs.

The accuracy of the proposed method was evaluated in a 6-point matrix standard addition experiment. A urine pool was spiked with 10, 20, 50, 100, 500 and 1000 pg/mL of standards (four times higher spike for 1- and 2-NAP). The un-spiked and spiked urine pools were analyzed, each in six replicates. A linear regression analysis was carried out by plotting the measured concentrations against spiked concentrations to evaluate correlations and determine concentrations of analyte in the non-spiked urine sample. As shown in Table 2, the matrix-spiked samples gave good linearity for all compounds with correlation coefficients ranging 0.92–1.00. The intercept from the linear regression reflected 80–109% of the measured concentrations in the un-spiked urine pool and the differences were not statistically significant (alpha = 0.05), demonstrating a non-biased and accurate method.

The accuracy of this method was further evaluated through two NISTSRMs, namely SRM 3672 (smoker urine) and SRM 3673 (non-smoker urine). As shown in Table 3, the results on OH-PAHs from this method were in good agreement with the certified concentrations (Draft Certificates of Analysis, internal communication with Dr. Michele Schantz, NIST) that spanned several orders of magnitude, further demonstrating the accuracy of the proposed method.

Analysis of smoker and non-smoker samples

Table 4 lists the geometric mean (GM) and selected percentile urinary concentrations in 30 self-identified smokers and 30 non-smokers, as well as the OH-PAH concentrations in the general, non-smoking and smoking U.S. population [36]. As expected, OH-PAH concentrations were on average 9.0 (range: 3.9–25) fold higher in smokers than non-smokers. For example, the GM concentrations of 1-PYR, the most commonly used PAH exposure biomarker, were 58 and 482 ng/L in the non-smoker and smoker samples, respectively, corresponding to 8.3 fold difference between these two groups. Me-OHNs were detected in 53–97% of non-smokers and 100% of smokers. As shown in Table 4, the geometric mean concentrations for Me-OHNs ranged from 34–453 pg/mL in the non-

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smokers to 1,608–6,990 pg/mL in the smokers, which corresponds to an average of 36.7 (range: 9.9–61) fold difference.

Interestingly, the concentration differences between smokers and non-smokers of Me-OHNs are even higher than those of OH-PAHs, the commonly recognized PAH exposure biomarkers. In the 30 non-smokers, the GM urinary concentrations of MeN metabolites (sum of 11 Me-OHNs; 1.7 ng/mL) were lower than those of the naphthalene metabolites (sum of 1- and 2-naphthol; 5.8 ng/mL). In the 30 smokers, the GM concentrations of the metabolites from naphthalene and MeNs were comparable, at 33.7 and 33.4 ng/mL, respectively (Supplementary Material, Figure S5). Similarly, the Me-OHN concentrations were 10.9–30.0 times higher in the NIST smoker urine SRM than the non-smoker urine SRM, while for the OH-PAHs, the differences were smaller (0.2–11.0 fold). These results were consistent with the report that a higher amount of methyl PAHs, such as MeNs, were generated in tobacco smoking compared to other combustion sources [4, 5], and strongly suggested the utility of urinary Me-OHNs as biomarkers to assess human exposure to MeNs.

Conclusions

To the best of our knowledge, this is the first method reporting the concurrent measurement of 11 metabolites of MeNs and 10 metabolites of un-substituted PAHs in 1 mL of human urine with optimal precision, accuracy and sensitivity. The automated sample preparation permits robust operation and high throughput, essential for the analysis of samples in national surveys and other large epidemiological studies. We evaluated the usefulness of the method to assess exposure to PAHs by analyzing urine specimens from smokers and non-smokers, two populations known to have different level of PAH exposure. It should be noted that many factors, such as urine dilution, demography, geography and timing of sample collection, can affect metabolite concentrations. Nonetheless, the magnitude of concentration differences between smokers and non-smokers, especially for Me-OHNs, strongly suggest the utility of these compounds as potential biomarkers for assessing exposure to PAHs, MeNs and related sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PAH	Polycyclic aromatic hydrocarbon
MeN	Methylnaphthalene
BaP	Benzo[*a*]pyrene

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Me-OHN	Methylnaphthol
OH-PAH	Monohydroxy polycyclic aromatic hydrocarbon
GC-MS/MS	Gas chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
QC	Quality control materials
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
1-PYR	1-Hydroxypyrene
3-BaP	3-Hydroxybenzo[*a*]pyrene
^{13}C-IS	^{13}C-labeled internal standard
^{13}C-PCB105	$^{13}C_{12}$-labeled 2,3,3′,4,4′-pentachlorobiphenyl
CDC	Centers for Disease Control and Prevention
SRM	Standard reference material
NIST	National Institute of Standard and Technology
MRM	Multiple reaction monitoring
GM	Geometric mean

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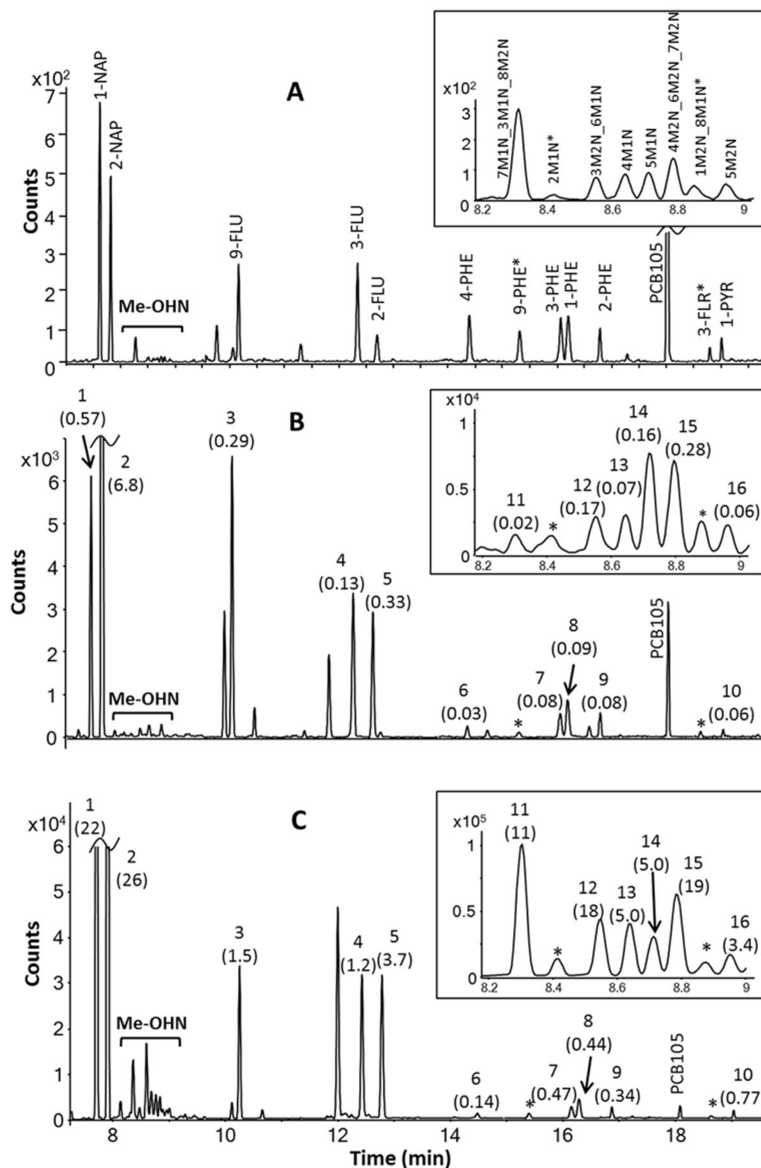

Figure 1.

Gas chromatograms of (A) a standard (1 pg injection on column for 1- and 2-NAP, 250 fg for other analytes), and urine extracts from a non-smoker (B) and a smoker urine sample (C). Peaks represent the trimethylsilyl ethers of OH-PAHs and Me-OHNs. Peaks marked with an asterisk are isomers that are not reported in this method. Calculated concentrations in urine samples (ng/mL) are given in parenthesis.

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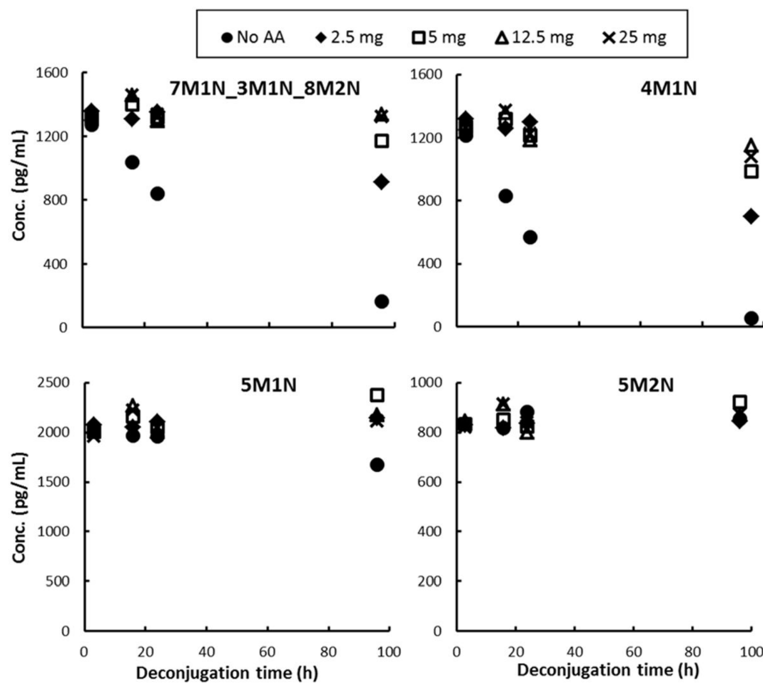

Figure 2.

Calculated concentrations of selected methyl naphthols in a smoker's urine sample as a function of deconjugation time for 5 different amounts of ascorbic acid (AA, mg/mL urine)

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Table 1

GC-MS/MS parameters for target analytes and corresponding ^{13}C-labeled internal standards

Analytes	Target analytes				^{13}C-labeled internal standard			
	Abbr.	Ion transition (m/z)	Collision energy (eV)	R.T. (min)	Abbr.	Ion transition (m/z)	Collision energy (eV)	R.T. (min)
Naphthalene metabolites								
1-OH-naphthalene[a]	1-NAP	216.1→185.1	27	9.0	^{13}C6-1-NAP[e]	222.1→191.2	27	9.0
2-OH-naphthalene[a]	2-NAP	216.1→185.1	27	9.2	^{13}C6-2-NAP[e]	222.1→191.2	27	9.2
Fluorene metabolites								
9-OH-fluorene[a]	9-FLU	254.1→165.0	23	11.0	^{13}C6-9-FLU[e]	260.1→170.9	23	11.0
3-OH-fluorene[a]	3-FLU	254.1→165.0	20	12.2	^{13}C6-3-FLU[e]	260.1→170.9	30	12.2
2-OH-fluorene[a]	2-FLU	254.1→165.0	20	12.5	^{13}C6-2-FLU[f]	260.1→170.9	30	12.5
Phenanthrene metabolites								
4-OH-phenanthrene[b]	4-PHE	234.8→220.0	30	13.4	^{13}C4-4-PHE[e]	270.1→238.8	30	13.4
3-OH-phenanthrene[c]	3-PHE	266.5→235.2	27	14.2	^{13}C6-3-PHE[e]	272.1→241.2	27	14.2
1-OH-phenanthrene[b]	1-PHE	266.5→235.2	27	14.3	^{13}C4-1-PHE[e]	270.1→239.2	27	14.3
2-OH-phenanthrene[b]	2-PHE	266.5→235.2	27	14.8	^{13}C6-2-PHE[e]	272.1→241.2	27	14.8
Pyrene metabolites								
1-OH-pyrene[a]	1-PYR	290.1→258.9	30	17.5	^{13}C6-1-PYR[e]	296.1→265.2	30	17.5
1-Methyl naphthalene metabolites								
8-Methyl-2-naphthol[d]	8M2N	229.9→200.0	20	9.7[g]	^{13}C6-2M1N[e]	236.1→206.0	20	9.8
4-Methyl-1-naphthol[d]	4M1N	229.9→200.0	20	9.95				
5-Methyl-1-naphthol[d]	5M1N	229.9→200.0	20	10.0				
4-Methyl-2-naphthol[d]	4M2N	229.9→200.0	20	10.1[h]				
5-Methyl-2-naphthol[d]	5M2N	229.9→200.0	20	10.2				
2-Methyl naphthalene metabolites								
3-Methyl-1-naphthol[d]	3M1N	229.9→200.0	20	9.7[g]	^{13}C6-2M1N[e]	236.1→206.0	20	9.8
7-Methyl-1-naphthol[d]	7M1N	229.9→200.0	20	9.7[g]				
3-Methyl-2-naphthol[d]	3M2N	229.9→200.0	20	9.9[i]				

Analytes	Target analytes				^{13}C-labeled internal standard			
	Abbr.	**Ion transition (m/z)**	**Collision energy (eV)**	**R.T. (min)**	**Abbr.**	**Ion transition (m/z)**	**Collision energy (eV)**	**R.T. (min)**
6-Methyl-1-naphthol[d]	6M1N	229.9→200.0	20	9.9[i]				
6-Methyl-2-naphthol[d]	6M2N	229.9→200.0	20	10.1[h]				
7-Methyl-2-naphthol[d]	7M2N	229.9→200.0	20	10.1[h]				

[a]Purchased from Sigma-Aldrich, St. Louis, MO, USA

[b]Purchased from LGC Promochem, Teddington, United Kingdom

[c]Purchased from Dr. Ehrenstorfer, Augsburg, Germany

[d]Purchased from Chiron AS, Trondheim, Norway

[e]Purchased from Cambridge Isotpe Laboratories (CIL), Andover, MA, USA

[f]Purchased from Los Alamos National Laboratory, Los Alamos, NM, USA

[g]7-Methyl-1-naphthol, 3-Methyl-1-naphthol and 8-Methyl-2-naphthol coelute

[h]4-Methyl-2-naphthol, 6-Methyl-2-naphthol and 7-Methyl-2-naphthol coelute

[i]3-Methyl-2-naphthol and 6-Methyl-1-naphthol coelute

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Table 2

Method validation parameters, including method detection limit (MDL), precision of quality control (QC) characterizations and matrix-spiked standard addition parameters.

Analyte	MDL* (pg/mL)	Low QC (n=30)				High QC (n=30)				Matrix-Spike (6-level)			
		Mean (pg/mL)	Between day SD (pg/mL)	Within day SD (pg/mL)	Overall CV	Mean (pg/mL)	Between day SD (pg/mL)	Within day SD (pg/mL)	Overall CV	Slope	Intercept (pg/mL)	r^2	unspiked Conc (pg/mL)
1-NAP	2.8	812	55	27	6.3%	1230	111	59	8.3%	1.00	868	1.00	901
2-NAP	8.7	1810	112	65	5.6%	2270	153	102	5.9%	0.98	1210	1.00	1200
9-FLU	1.8	310	23	9	7.3%	628	48	24	7.1%	1.01	247	1.00	248
3-FLU	19	268	22	9	7.9%	689	56	28	7.6%	1.02	257	1.00	255
2-FLU	1.2	465	28	12	5.6%	789	85	31	10%	1.02	408	1.00	406
4-PHE	1.1	317	18	9	5.2%	773	42	31	4.7%	0.87	10	1.00	11
3-PHE	3.1	273	17	9	5.8%	704	40	31	4.8%	0.92	124	1.00	122
1-PHE	1.5	274	17	10	5.7%	691	45	31	5.6%	0.98	78	1.00	82
2-PHE	1.0	210	29	8	13%	596	81	25	13%	0.97	54	1.00	56
1-PYR	5.1	348	26	17	6.5%	906	71	58	6.4%	1.02	109	0.99	108
7M1N_3M1N_8M2N	18	727	99	49	13%	1850	302	99	16%	1.16	290	0.99	302
3M2N_6M1N	41	588	82	51	12%	1060	173	80	15%	1.15	763	0.97	754
4M1N	9.3	400	56	29	13%	992	165	68	16%	1.18	139	0.99	147
5M1N	7.0	702	103	54	14%	1690	242	109	14%	1.23	352	0.97	333
4M2N_6M2N_7M2N	13	1770	268	120	14%	3980	581	267	14%	1.23	834	0.97	779
5M2N	19	609	76	44	11%	1740	226	130	12%	1.27	134	0.98	123

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Table 3

Mean OH-PAH and Me-OHN urinary concentrations in pg/mL (standard deviation and coefficients of variation from 4 repeated measurements) in Standard Reference Materials (SRMs).

No	Analyte	SRM 3672 Smoker urine		SRM 3673 Non-smoker urine	
		This method	Certified Conc.*	This method	Certified Conc.*
1	1-NAP	33,900 (275, 0.8%)	34,400	197,000 (3,140, 1.6%)	211,000
2	2-NAP	8,770 (70, 0.8%)	8,730	1,340 (23, 1.7%)	1,350
3	9-FLU	357 (9, 2.4%)	337	109 (2, 1.6%)	110
4	3-FLU	404 (7, 1.7%)	428	35 (0.4, 1.1%)	39
5	2-FLU	823 (24, 2.9%)	870	94 (3, 3.7%)	107
6	4-PHE	36 (1, 3.3%)	49	8 (1, 11%)	10
7	3-PHE	97 (2, 2.1%)	125	20 (1, 4.1%)	28
8	1-PHE	141 (2, 1.6%)	136	50 (2, 3.5%)	49
9	2-PHE	89 (2, 2.7%)	84	23 (2, 6.7%)	25
10	1-PYR	201 (5, 2.5%)	173	32 (2, 6.4%)	30
11	7M1N_3M1N_8M2N	3,080 (207, 6.7%)	-	109 (10, 9.3%)	-
12	3M2N_6M1N	4,400 (240, 5.5%)	-	244 (14, 5.8%)	-
13	4M1N	2,000 (185, 9.2%)	-	84 (3, 3.1%)	-
14	5M1N	2,120 (94, 4.4%)	-	86 (2, 2.7%)	-
15	4M2N_6M2N_7M2N	6,290 (375, 6.0%)	-	571 (32, 5.6%)	-
16	5M2N	951 (36, 3.8%)	-	23 (5, 20%)	-

*Certified concentrations on OH-PAHs were obtained from the SRMs' draft Certificate of Analysis (COA, internal communication with Dr. Michele Schantz, NIST). Mass fraction concentrations (μg/kg) were converted to urinary concentration (pg/mL) using a urine density value of 1.019 g/mL, as specified on the COAs.

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Table 4

Geometric mean (GM) and select percentile concentrations (pg/mL) of OH-PAHs and Me-NAPs in 30 non-smokers and 30 smokers. Also given are GMs in the U.S. adults and least square geometric means (LSGM) in U.S. non-smokers and smokers.

No	Analyte	Non-smokers (n=30)				Smokers (n=30)				US adults (20 yr)*		
		GM	25th	50th	75th	GM	25th	50th	75th	GM	LSGM Non-smoker	LSGM Smoker
1	1-NAP	1760	510	911	1280	12400	9640	14100	21300	2190	1520	6290
2	2-NAP	2130	1310	1930	2700	19900	12500	20800	32500	2620	1680	8600
3	9-FLU	243	100	189	603	1252	750	1126	1928	230	200	342
4	3-FLU	52	24	48	78	1321	968	1429	2168	138	90	592
5	2-FLU	138	68	133	205	2000	1440	2030	2930	333	236	990
6	4-PHE	16	<LOD	15	29	85	54	92	119	43	39	53
7	3-PHE	53	22	49	84	380	275	348	592	105	91	194
8	1-PHE	84	38	84	144	330	241	326	452	145	132	193
9	2-PHE	42	16	38	70	169	103	169	245	56	48	88
10	1-PYR	58	22	47	94	482	288	425	872	47	40	104
11	7M1N_3M1N_8M2N	152	60	148	272	4920	3690	4540	11600	-	-	-
12	3M2N_6M1N	419	245	375	653	5660	3530	5560	9570	-	-	-
13	4M1N	123	64	114	219	6990	4840	8090	12700	-	-	-
14	5M1N	106	47	100	313	6420	4270	8200	12900	-	-	-
15	4M2N_6M2N_7M2N	453	201	404	1760	4490	2980	4770	8380	-	-	-
16	5M2N	34	<LOD	23	81	1610	1030	1980	3220	-	-	-

* Geometric mean in US adults, and least square geometric means in US non-smokers and smokers are referenced from [36].