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Determination of Phenolic Metabolites of Polycyclic Aromatic Hydrocarbons in Human Urine as Their Pentafluorobenzyl Ether Derivatives Using Liquid Chromatography−Tandem Mass Spectrometry

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, and a number of them are carcinogenic. One approach for measuring exposure to them is to determine the concentrations of metabolites in urine. The pyrene metabolite 1-hydroxypyrene has been used as a biomarker for exposure in numerous studies. However, determination of exposure to several PAHs may be advantageous, since the relative amounts may vary depending upon the exposure source. We developed a liquid chromatography−tandem mass spectrometry method for the determination of phenolic metabolites of naphthalene, fluorene, phenanthrene, and pyrene in human urine. Following enzymatic cleavage of the glucuronide and sulfate conjugates, the phenolic metabolites are extracted from urine and converted to pentafluorobenzyl ethers. These derivatives greatly enhance the sensitivity of detection by atmospheric pressure chemical ionization in the negative ion mode. Lower limits of quantitation range from 0.01 to 0.5 ng/mL. Stable isotope-labeled internal standards were synthesized or obtained commercially. Data on urinary excretion of several PAH metabolites in urine of smokers and nonsmokers are presented.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, arising from numerous combustion sources, including motor vehicles, fireplaces, tobacco smoke, power plants, and various other industrial sources.[1,2] In addition, diet is a major source of human exposure to these substances, including the relatively volatile naphthalene, fluorene, and phenanthrene, as well as less volatile PAHs such as pyrene and benzo[*a*]pyrene, with four or more fused rings.[3−5] Since a number of PAHs are carcinogenic, there has been a great deal of interest in developing methods for determining human exposure. Generally the higher molecular weight PAHs, such as benzo[*a*]pyrene, are the most carcinogenic, but recent studies have concluded that naphthalene is a substance that contributes considerably to human cancer risk.[4] A widely used approach for assessing exposure is to measure concentrations of PAH metabolites in urine.[6] Since urinary excretion of metabolites of highly carcinogenic PAHs such as benzo[*a*]pyrene is very low and variable due to extensive elimination in the feces, and therefore difficult to quantify,[6] "surrogate markers" are generally used. The pyrene metabolite 1-hydroxypyrene (1-HP) has been used as a biomarker for PAH exposure in numerous studies.[7] However, since ratios of various PAHs may vary depending upon the source,[8−11] measuring concentrations of metabolites of several different PAHs may be advantageous in studies of exposure.[12−16] Liver enzyme induction can affect metabolite ratios, so measuring concentrations of several metabolites might be used to evaluate induction of cytochrome

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P450 (CYP) enzymes.[17−21] For example, it has been reported that the ratio of 1- + 2-hydroxyphenanthrene/3- + 4-hydroxyphenanthrene is lower in smokers than in nonsmokers, as a result of induction of 3,4-oxidation catalyzed by CYP1A2.[17,18] Hydroxylated PAH metabolites are excreted mainly as their glucuronide conjugates, but varying amounts of sulfate conjugates and small amounts of free phenols may also be excreted.[6,22]

Most of the methods that have been used to determine phenolic metabolites of PAHs, in particular 1-HP, have employed high-performance liquid chromatography (HPLC) with fluorescence detection.[6,7,15,18,23] An advantage of these methods is that HPLC instruments are available in many laboratories and that fluorescence detection is highly sensitive and can provide fairly good specificity for determining PAH metabolites. Disadvantages are that often fairly large urine specimens (10 mL or more) are needed, long HPLC run times may be required, and most methods do not employ an internal standard, which, if used, would be expected to result in better precision and accuracy.[15,23,24]

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) methods for determination of phenolic PAH metabolites have also been reported. An advantage of these methods is that capillary GC columns can provide better resolution of isomeric metabolites than HPLC columns.[25] Recently, GC-high-resolution MS methods for simultaneous determination of several PAH metabolites was reported; an advantage is the high sensitivity and specificity attainable on a high-resolution instrument.[26−28] A disadvantage of GC/MS methods compared to HPLC-fluorescence is that derivatization of the analytes is necessary and that more expensive instruments are required, especially in the case of high-resolution mass spectrometers, which are not widely available.

In recent years, liquid chromatography/mass spectrometry (LC/MS) instruments have become available in many laboratories, a major driving force being their widespread use in drug discovery and development.[29] With LC coupled to triple-stage quadrupole tandem mass spectrometers (LC/MS/MS), a wide range of substances in complex biological matrixes can be quantitated at low levels and with high specificity, often with short chromatographic run times.[29,30] Despite this, relatively few LC/MS methods have been reported for determination of hydroxylated metabolites of PAHs. Determination of 1-HP in urine using LC- time-of-flight MS with electrospray ionization (ESI),[31] and by LC/MS/MS with ESI,[32,33] have been reported, and an LC/MS method that utilized a single quadrupole instrument with ESI has been reported for determination of phenolic metabolites of PAHs from in vitro metabolism studies.[34] LC/MS methods using atmospheric pressure chemical ionization (APCI) or ESI for determination of various hydroxylated PAHs have been reported but not applied to biological samples.[35−38] Recently, an LC-ESI-MS/MS method for selective detection and preliminary quantification of certain monohydroxy PAH metabolites in human urine was reported, including data on excretion of metabolites of the potent carcinogens benzo[*a*]pyrene and benz[*a*]anthracene.[39] LC-APCI-MS/MS determination of 1-HP and 3-hydroxybenzo[*a*]pyrene has been reported,[40] but the sensitivity was adequate only for urine from persons with relatively high levels of PAH exposure.[16]

As part of our studies of toxic substance exposure in people smoking cigarettes of different composition, we needed a method for the determination of several PAH metabolites in human urine that would be suitable for large numbers of samples generated in clinical studies. In this paper, we describe an LC/MS/MS method for determination of monophenolic metabolites of naphthalene, fluorene, phenanthrene, and pyrene (Figure 1). The method

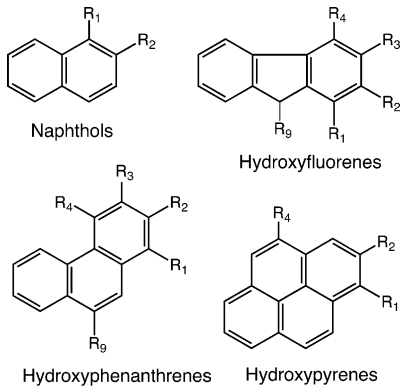

Figure 1. Ring-hydroxylated PAH metabolites. The subscripts of the R groups indicate the ring positions of the hydroxy groups for the monophenolic metabolites, e.g., for 2-hydroxyfluorene R_2 = OH and all other R groups = H.

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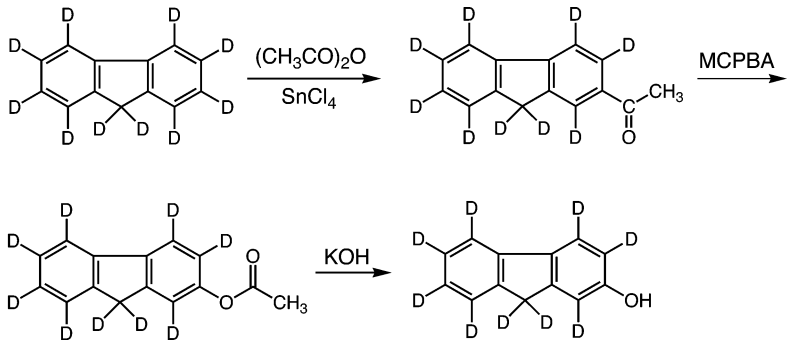

Figure 2. Synthesis of 2-hydroxyfluorene-d_9. A mixture of hydroxyphenanthrene isomers was synthesized analogously. MCPBA = *m*-chloroperbenzoic acid.

Table 1. Parent Ions, Product Ions, and Collision Energies for Analytes and Internal Standards

analyte or internal standard	parent mass	product mass	collision energy, eV
2-naphthol	143	115	30
2-naphthol-d_6	149	121	30
1-hydroxyfluorene	181	153	28
2-hydroxyfluorene	181	180	31
3-hydroxyfluorene	181	153	28
2-hydroxyfluorene-d_9	190	162	28
2-hydroxyfluorene-d_9	190	188	31
hydroxyphenanthrenes	193	165	38
hydroxyphenanthrenes-d_9	202	174	38
1-hydroxypyrene	217	189	22
1-hydroxypyrene-$^{13}C_6$	223	195	22

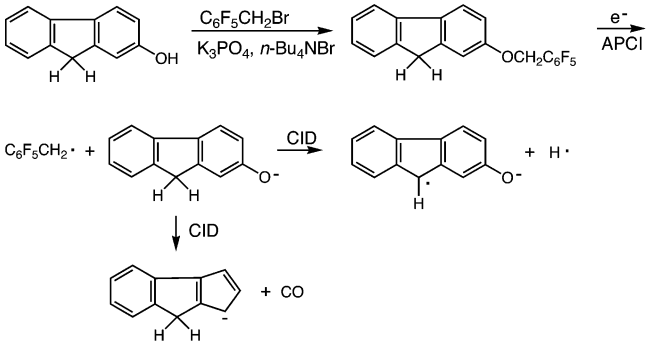

Figure 3. Conversion of phenolic metabolites to PFB derivatives and proposed fragmentation pathways. Illustrated for 2-hydroxyfluorene pentafluorobenzyl ether.

involves conversion of the metabolites to the pentafluorobenzyl ether derivatives, in order to enhance sensitivity by making use of the recently developed electron capture atmospheric pressure chemical ionization (ECAPCI) technique.[41] Advantages of this method include simultaneous determination of several analytes with good precision and accuracy, very high sensitivity, an extraction/derivatization procedure suitable for processing large batches of samples, and a relatively short (15 min) instrument run time.

EXPERIMENTAL SECTION

Instrumentation. LC/MS and LC/MS/MS analyses were carried out with a Surveyor HPLC interfaced to a TSQ Quantum Ultra triple-stage quadrupole mass spectrometer (Thermo-Finnigan, San Jose, CA). A PAH Green column (4.6 × 150 mm, Thermo-Hypersil-Keystone (Bellefonte, PA) was used for the chromatography. For GC/MS and GC/MS/MS analyses, a Thermo Trace 2000 GC (Milan, Italy) with a 0.25 mm i.d. × 25 m DB-5 column (J & W Scientific, Folsom, CA) interfaced to a Finnigan TSQ 7000 mass spectrometer was used. Solvent evaporation was carried out using a Savant Automatic Environmental SpeedVac model AES 2000 (Thermo-Savant, Marietta, OH)

Chemicals. 1- and 2-naphthols and 1-hydroxypyrene were obtained from Acros Organics/Fisher Chemical Co. (Pittsburgh, PA). 1-Hydroxyfluorene was obtained from ChemBridge Corp. (San Diego, CA), and 2- and 3-hydroxyfluorene were from Aldrich Chemical Co. (Milwaukee, WI). 1- and 2-naphthols and 2-hydroxyfluorene were sublimed under reduced pressure prior to use. 1-, 2-, 3-, 4-, and 9-hydroxyphenanthrenes were purchased from Crescent Chemical Co. (Islandia, NY). 1-Hydroxypyrene-$^{13}C_6$ was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Pentafluorobenzyl bromide was obtained from Acros Organics/Fisher Chemical Co. 2-Naphthol-d_6, 2-hydroxyfluorene-d_9, and hydroxyphenanthrene-d_9 isomers were synthesized in our laboratory (Figures 2, S-1, and S-2, Supporting Information). The syntheses are described in the Supporting Information. HPLC grade methanol and water from Burdick and Jackson (Muskegon, MI) were used to prepare the LC mobile phase. HPLC grade pentane and ethyl acetate from Fisher were used for extractions.

Preparation of Standards and Controls. An "artificial urine" was used as a matrix for the standards, prepared from major components reported in human urine.[42] Concentrations in grams per liter of deionized water were as follows: human albumin, 0.1; ammonium sulfate, 1.0; andosterone, 0.02; ascorbic acid, 0.02; bilirubin, 0.005; citric acid, 0.5; creatine, 0.5; creatinine, 1.5; cystine, 0.1; glucuronic acid, 0.5; hippuric acid, 1.0; histidine, 0.5; magnesium sulfate, 1.0; phenol, 0.5; potassium phosphate, monobasic, 1.0; sodium chloride, 10; urea, 15; uric acid, 0.5. The pH of the artificial urine solution was adjusted to between 5 and 6, and it was stored at −20 °C. Standards (including blanks) were prepared on the day of use by diluting eight stock methanolic solutions of the analyte standards 30-fold with the artificial urine. Controls were prepared by spiking pooled nonsmokers' urine with analyte stock solutions in methanol. A stock solution of internal standards was prepared by adding 100 μL of 1 mg/mL 2-naphthol-d_6, 50 μL of 1

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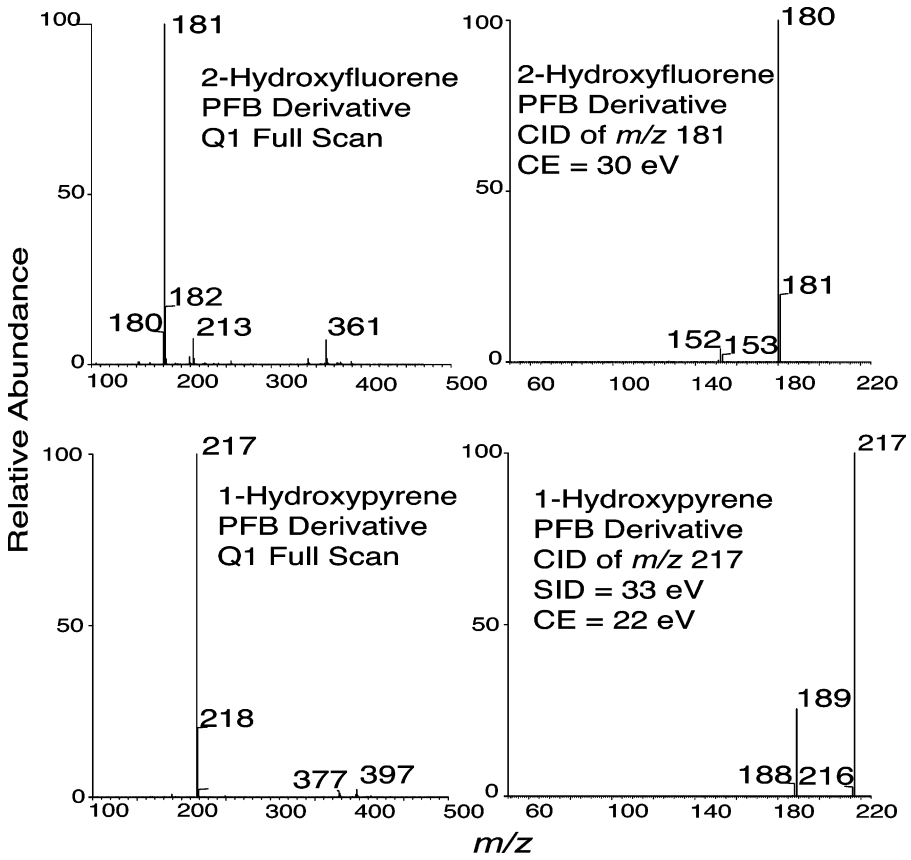

Figure 4. APCI spectra of 2-hydroxyfluorene and 1-hydroxypyrene pentafluorobenzyl ethers.

mg/mL 2-hydroxyfluorene-d_9, 50 μL of 1 mg/mL hydroxyphenanthrene-d_9 isomers (method A, Supporting Information), 60 μL of 1 mg/mL hydroxyphenanthrene-d_9 isomers (method B, Supporting Information), and 1 mL of 10 μg/mL 1-hydroxypyrene-$^{13}C_6$ to 125 mL of methanol. This composition was chosen to give MS responses comparable to those of the natural isotopomers in smokers and to give similar peak areas of the isomeric deuterium-labeled hydroxyphenanthrenes.

Sample Preparation. The internal standard solution (50 μL) was added to urine specimens, standards, blanks, or controls (2.7 mL), which were buffered to pH 7 with 0.3 mL of 1 M phosphate buffer and incubated overnight at 37 °C with β-glucuronidase (3000 units, type 1XA from *Eschericheria coli*, Sigma, St. Louis, MO) and sulfatase (0.6 unit, type VI from *Aerobacter aerogenes*, Sigma). The samples were extracted with a 90:10 mixture (v/v) of pentane/ethyl acetate (4 mL) by vortex mixing, and the phases were separated by centrifuging (~1000*g*) and freezing the aqueous layers in a dry ice/acetone bath. The organic phases were poured into tubes containing 150 μg of gallic acid in 30 μL of methanol, and the solvent was removed using a centrifugal vacuum evaporator at ambient temperature. To the residues were added pentafluorobenzyl bromide (100 μL of 5% in methylene chloride), aqueous tetrabutylammonium bromide (50 μL of 5%), and aqueous tripotassium phosphate (50 μL of 20%). The tubes were capped, vortex-mixed for 30 min, and then vortexed with 100 μL of ammonium hydroxide (10% in 40/60 water/methanol) to destroy excess pentafluorobenzyl bromide. After adding 1 mL of 4 M sulfuric acid, the derivatives were extracted with pentane (3 mL, vortex, centrifuge, freeze/pour) and evaporated to dryness. (Subsequent to method validation, we found that the amount of PFBBr could be reduced to 20 μL of 5%, and the ammonia and sulfuric acid steps could be omitted, with about 20−50% improvement in sensitivity.) The residues were dissolved in 120 μL of methanol, and 20 μL was injected into the LC/MS/MS system. Extraction efficiency was determined by analyzing 10 urine specimens with the labeled internal standards added prior to enzyme treatment and extraction, as well as aliquots of the same specimens to which internal standards were added after extraction. The differences in mean peak areas for samples with internal standards added prior to extraction and those for which internal standards were added after extraction were used to calculate extraction recoveries. These were as follows: 2-naphthol-d_6, 72%; 1-hydroxyphenanthrene-d_9, 73%, 2-hydroxyphenanthrene-d_9, 82%; 3- + 4-hydroxyphenanthrene-d_9, 80%; 2-hydroxyfluorene-d_9, 81%; 1-hydroxypyrene-$^{13}C_6$, 83%. *Note:pentafluorobenzyl bromide is a potent lachrymator, and it should be handled in an efficient fume hood.*

Liquid Chromatography. The analytes were separated using a gradient of water and methanol, 0.7 mL/min, with the following program: initial 80% methanol, 0.5 min 90% methanol, 5 min 94% methanol, 5.5−9 min 100% methanol, and 9.1−15 min (end of run) 80% methanol.

Mass Spectrometry. The mass spectrometer was operated using APCI, in the negative ion mode. The ion source parameters were optimized by infusing a methanol solution of the pentafluorobenzyl (PFB) derivative of 2-hydroxyfluorene via syringe pump. The vaporizer temperature was 317 °C, the heated capillary temperature was 251 °C, and the corona discharge current was set at 50 μA. The PFB derivatives of the individual analytes were infused to determine appropriate ion transitions and the optimum

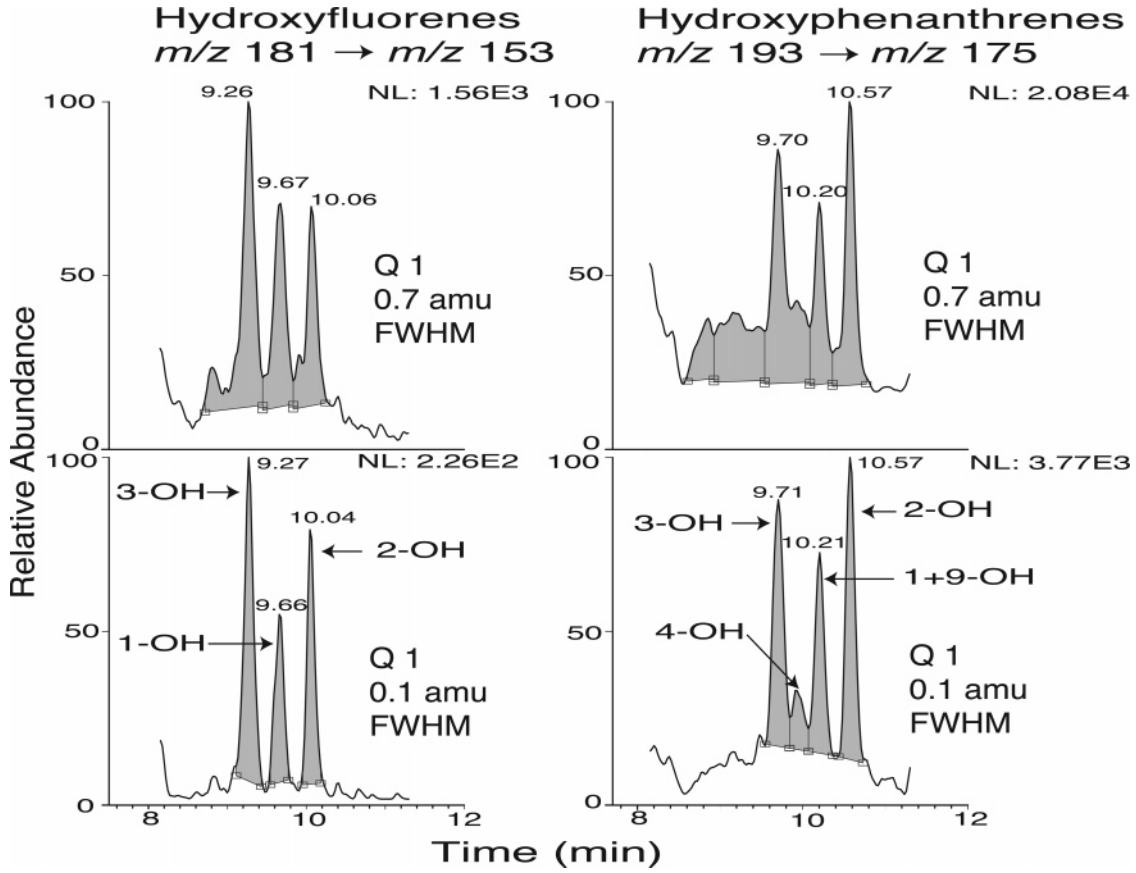

Figure 5. Effect of Q 1 mass resolution (Q 3 0.7 amu fwhm). LC/MS SRM chromatograms, hydroxyfluorenes and hydroxyphenanthrenes in extract of nonsmoker's urine.

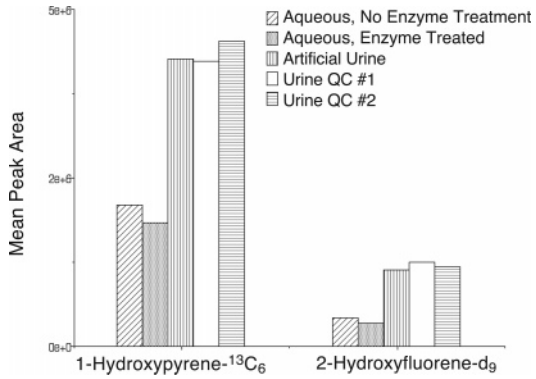

Figure 6. Relative recovery of 2-hydroxyfluorene-d_9 and 1-hydroxypyrene-$^{13}C_6$ from different sample matrixes. Urine and artificial urine were treated with enzyme β-glucuronidase/arylsulfatase.

collision energies for collision-induced dissociation (CID). The collision gas (argon) pressure was 1.5 mTorr. The parent and product ions and collision energies used for quantitation are in Table 1. Three acquisition segments were used. The PFB derivatives of 2-naphthol and 2-naphthol-d_6 eluted in the first; the PFB derivatives of the hydroxyfluorenes, hydroxyphenanthrenes, and their deuterated isotopomers eluted in the second; 1-hydroxypyrene and 1-hydroxypyrene-$^{13}C_6$ PFB derivatives eluted in the third. Source CID collision energy was 33 eV for 1-hydroxypyrene and 1-hydroxypyrene-$^{13}C_6$. Source CID was not used for the other analytes. For all analytes, Q1 peak width was 0.1 amu fwhm, and Q3 peak width was 0.7 amu fwhm.

Data Analysis. The Finnigan XCalibur/LC Quan software was used to generate calibration curves (linear regression, $1/X$ weighting) and calculate concentrations using peak area ratios of analyte/internal standard. The ISTD for 2-naphthol was 2-naphthol-d_6, the ISTD for 1- and 3-hydroxyfluorenes was 2-hydroxyfluorene-d_9 (m/z 190 > m/z 162), the ISTD for 2-hydroxyfluorene was 2-hydroxyfluorene-d_9 (m/z 190 > m/z 188), the ISTD for 3- and 4-hydroxyphenanthrene was 3-hydroxyphenanthrene-d_9 (first eluting), the ISTD for 1-hydroxyphenanthrene was 1-hydroxyphenanthrene-d_9 (and/or 9- hydroxyphenanthrene-d_9, second eluting), the ISTD for 2-hydroxyphenanthrene was 2-hydroxyphenanthrene-d_9 (third eluting), and the ISTD for 1-HP was 1-HP-$^{13}C_6$ (Table 1). Eight standards were used, at concentrations spanning the expected range, 0.25−50 ng/mL for 2-naphthol and from 0.01 to 5 ng/mL for all other analytes. "Artificial urine" blanks were included in all runs. The following analyte pairs were not resolved and were reported as sums: 1- and 2-naphthols; 1- and 9-hydroxyphenanthrenes; 3- and 4-hydroxyphenanthrenes. Correlation coefficients (r^2) for typical calibration curves are as follows: 2-naphthol, 0.9960; 1-hydroxyfluorene, 0.9989; 2-hydroxyfluorene, 0.9974; 3-hydroxyfluorene, 0.9739; 1-hydroxyphenanthrene, 0.9995; 2-hydroxyphenanthrene, 0.9988; 3- + 4-hydroxyphenanthrenes, 0.9998; 1-hydroxypyrene, 0.9996.

Clinical Samples. Urine specimens were obtained from smokers and nonsmokers participating in research studies, which were approved by the Institutional Review Board at the University of California, San Francisco, and were stored frozen at −20 °C prior to analysis. Stability of stored urine samples was evaluated

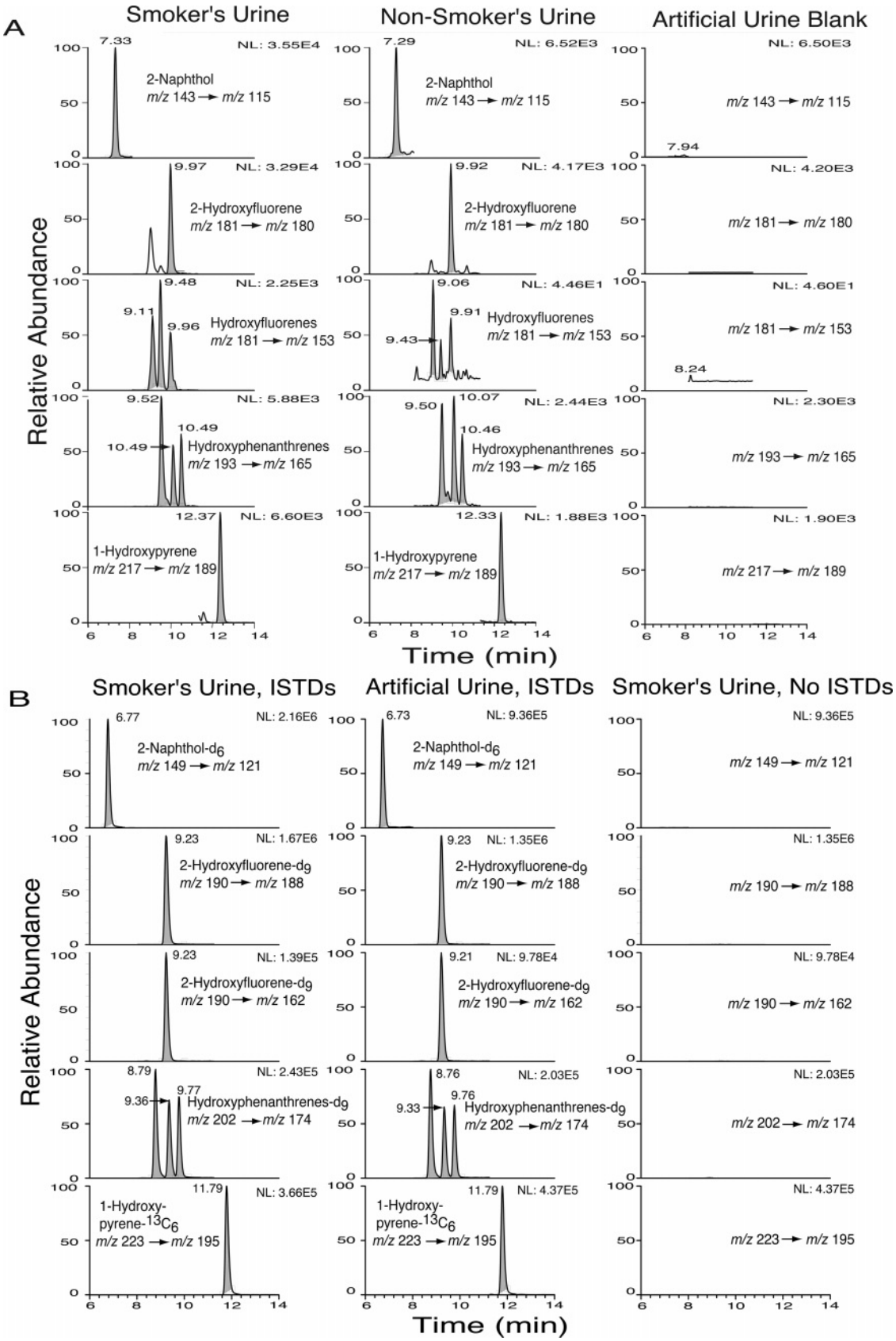

Figure 7. LC/MS SRM chromatograms of urine extracts. Q 1 resolution 0.1 amu fwhm, Q3 resolution 0.7 amu fwhm. (A) SRM transitions for analytes; (B) SRM transitions for internal standards.

by reanalyzing samples 1 year after the original analysis. The means (six nonsmokers and seven smokers) of the percent differences were within ±15% of the original analytical results (Table S-1, Supporting Information).

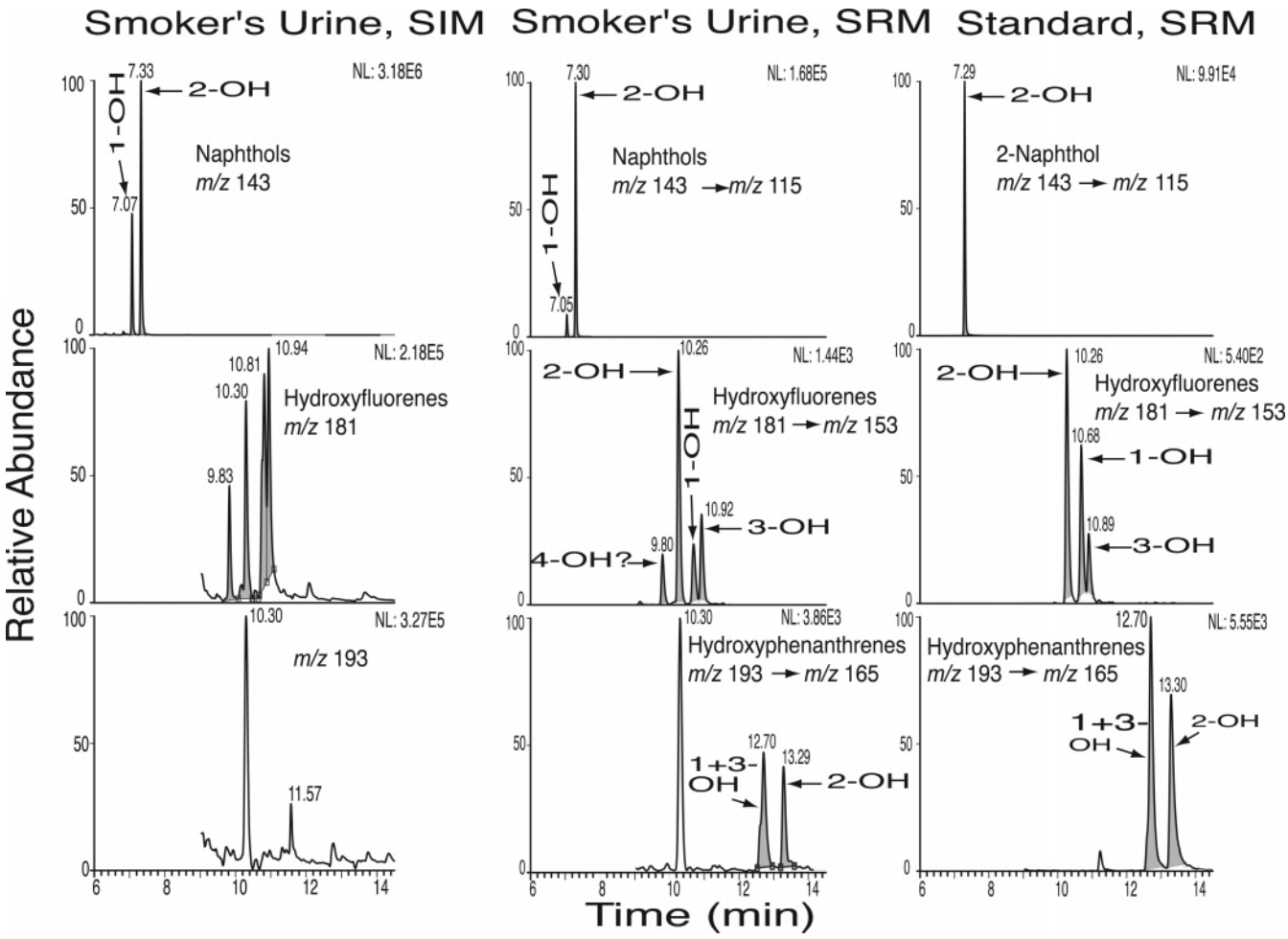

Figure 8. GC/MS chromatograms, methane NICI.

RESULTS AND DISCUSSION

API Mass Spectrometry. Initially, we attempted to develop a method for determining the phenolic metabolites without conversion to a derivative. Using APCI or ESI ionization modes and single-stage MS, 1-HP was converted to the expected $(M - H)^-$ ion, m/z 217. Operating the instrument in the MS/MS mode, CID converted the $(M - H)^-$ ion to the major product ion m/z 189, presumably via loss of CO. Using the transition m/z 217 to 189, we attempted to develop a selected reaction monitoring (SRM) method for 1-HP, but sensitivity with APCI appeared inadequate for determination of the subnanogram per milliliter levels that would be required. ESI was found to be more sensitive than APCI, but we were concerned that matrix suppression of ionization that often occurs with ESI might be a problem[39,43−47] and we did not evaluate this ionization mode with urine extracts. Using selected ion monitoring (SIM) of m/z 217, sensitivity for pure standards was better than when using SRM by 1 order of magnitude or more, but sensitivity still appeared to be marginal, and specificity would not be expected to be as good as that attainable using SRM. Indeed, considerable background ion current and extraneous peaks were encountered in SIM analysis of both standards and urine extracts. Therefore, we turned our attention to converting the PAH metabolites to PFB derivatives in order to use the highly sensitive ECAPCI technique.[41] 1-HP and the other phenolic PAH metabolites were readily converted to the PFB derivatives with pentafluorobenzyl bromide and a base. Conversion to derivatives was indicated by HPLC, and in the case of 2-naphthol, the structure was verified by GC/MS with electron ionization, which produced the molecular ion m/z 324 (17%), as well as fragments m/z 181 ($C_6F_5CH_2^+$, 78%) and 115 ($C_{10}H_{17}O^+$, 100%).

ECAPCI produced aryloxy anions by loss of the PFB radical from the initially generated radical anion. In ECAPCI, as well as negative ion chemical ionization (NICI) in GC/MS of PFB derivatives, the initially formed radical anions generally lose the PFB radical if a relatively stable anion can be formed.[41] Interestingly, applying source CID (also called in-source fragmentation) dramatically increased the ion current for some analytes, by 1 order of magnitude or more. Perhaps this aids in desolvation, or reduces the extent of protonation of the initially formed radical anion by methanol or water in the vaporized mobile phase, but we have no experimental evidence for these hypotheses. However, with the exception of 1-HP, source CID significantly increased

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Table 2. Precision and Accuracy, Spiked Pooled Nonsmokers' Urine, Intraday ($N = 7$)

analyte	added amount (ng/mL)	expected amount[a] (ng/mL)	measd mean (ng/mL)	accuracy (% of expected)	precision CV (%)
2-naphthol	0		1.43		8.0
	0.25	1.68	1.71	102	11
	1	2.43	2.64	109	4.2
	10	11.4	12.3	108	5.1
	50	51.4	52.1	101	6.0
LOQ (artificial urine)	0.500	0.505	0.518	103	7.6
1-hydroxyfluorene	0		0.038		19
	0.025	0.063	0.051	80.9	17
	0.1	0.138	0.128	92.9	8.4
	1	1.04	1.01	98.0	5.8
	5	5.04	4.83	95.7	9.4
LOQ (artificial urine)	0.100	0.105	0.106	101	19.5
2-hydrolxyfluorene	0		0.142		4.9
	0.025	0.167	0.177	106	5.1
	0.1	0.242	0.273	113	3.8
	1	1.14	1.20	105	4.3
	5	5.14	5.56	108	4.1
LOQ (artificial urine)	0.025	0.028	0.024	84	13
3-hydroxyfluorene	0		0.045		17
	0.025	0.070	0.056	81.0	29
	0.1	0.145	0.146	101	25
	1	1.05	1.22	116	10
	5	5.05	5.34	106	22
1-hydroxyphenanthrene	0		0.124		12
	0.025	0.149	0.148	99.1	9.2
	0.1	0.224	0.227	101	3.9
	1	1.12	1.117	99.3	5.9
	5	5.12	5.20	102	4.6
LOQ (artificial urine)	0.025	0.025	0.022	86	16
2-hydroxyphenanthrene	0		0.032		12
	0.025	0.057	0.057	99	7.4
	0.1	0.132	0.144	109	7.0
	1	1.03	1.05	101	5.9
	5	5.03	4.99	99.2	6.2
LOQ (artificial urine)	0.010	0.010	0.010	100	12
3- + 4-hydroxyphenanthrene[b]	0		0.096		7.5
	0.05	0.146	0.144	98.6	6.8
	0.2	0.296	0.292	98.6	3.8
	2	2.10	2.00	95.2	3.5
	5	5.10	4.87	95.5	3.2
LOQ (artificial urine)	0.020	0.020	0.019	96	10
1-hydroxypyrene	0		0.057		7.0
	0.025	0.082	0.082	100	2.0
	0.1	0.157	0.159	101	1.7
	1	1.06	0.97	91.8	3.4
	5	5.06	4.78	94.5	2.8
LOQ (artificial urine)	0.025	0.025	0.026	104	5.2

[a] Added amount + mean amount measured in blank urine (pool of three nonsmokers). [b] Spiked with equal amounts of 3- and 4-hydroxyphenanthrene. Relative response of 4-hydroxyphenanthrene was ~25% of the response of 3-hydroxyphenanthrene.

chemical noise observed in chromatograms derived from urine samples, so except for 1-HP, source CID was not used.

MS/MS spectra of the PFB derivatives of all analytes were recorded. With the exception of 2-hydroxyfluorene, the major product ions were $(M - PFB - 28)^-$ presumably formed by loss of CO, as was observed with the underivatized analytes. These transitions provided cleaner chromatograms of urine extracts than did the only reasonable alternatives, namely, the parent $(M - PFB)^-$ ions (in the SIM mode) or in the case of 1- and 3-hydroxyfluorene, the $(M - PFB - H)^-$ ion, m/z 180, or $(M - PFB - 28 - H)^-$ ion, m/z 152. For 2-hydroxyfluorene, the $(M - PFB - H)^-$ product ion provided much better sensitivity than the $(M - PFB - 28)^-$ ion, as well as excellent specificity. The ionization process and proposed structures for the product ions, illustrated for 2-hydroxyfluorene, are depicted in Figure 3. Spectra of the PFB derivatives of 1-hydroxypyrene and 2-hydroxyfluorene are shown in Figure 4. In addition to the major $(M - PFB)^-$ ions, $(M - H)^-$ ions were observed in low abundance, m/z 361 and 397, respectively. The ion transitions used for quantitation and collision energies are listed in Table 1. Sensitivity in the SRM mode was sufficient to detect a few picograms of derivatized standards.

Effect of Mass Resolution. The TSQ Quantum is capable of enhanced mass resolution, to 0.1 amu full width half-maximum (fwhm), without drastic loss in sensitivity. This can minimize interference from extraneous substances. For some analytes, operating the mass spectrometer at enhanced resolution (0.1 amu fwhm) greatly improved the specificity when applied to urine extracts. This was particularly evident for the hydroxyfluorenes and hydroxyphenanthrenes, for which high background ion

Table 3. Urine Concentrations of PAH Metabolites in Eighteen Smokers and Eight Nonsmokers Determined by LC/MS/MS and GC/MS/MS[a]

analyte	LC/MS/MS ng/mL mean (range)	GC/MS/MS ng/mL mean (range)	regression equation[b]	correlation coefficient
2-naphthol	10.5 (BLQ−51.1)	10.8 (BLQ −56)	$y = 1.08x - 0.547$	$r^2 = 0.965$
1-hydroxyfluorene	0.65 (BLQ −4.57)	0.61 (BLQ −3.49)	$y = 0.758x + 0.116$	$r^2 = 0.968$
2-hydroxyfluorene	1.12 (BLQ −6.62)	1.08 (0.047−6.29)	$y = 0.936x + 0.0265$	$r^2 = 0.993$
2-hydroxyphenanthrene	0.13 (BLQ −0.70)	0.10 (BLQ −0.57)	$y = 0.746x + 0.00448$	$r^2 = 0.976$

[a] BLQ, below the lower limit of quantitation. [b] Equation $y = Mx + B$, where y is the concentration by GC/MS/MS and x is the concentration by LC/MS/MS.

current and extraneous peaks made low-level quantitation difficult when the instrument was operated at unit resolution (0.7 amu fwhm). Much cleaner SRM chromatograms were obtained when Q1 was operated at 0.1 amu fwhm, thus minimizing interfering peaks and facilitating integration (Figure 5). For 2-hydroxyfluorene, the appearance of the chromatograms was further improved by operating Q3 at 0.1 amu resolution as well, in that the size of extraneous peaks were reduced (Figure S-3, Supporting Information). However, this did not appear to have a significant effect on analytical results and caused some loss in sensitivity, so in the analysis of biofluid samples Q1 resolution was set at 0.1 amu and Q3 resolution was set at 0.7 amu for all analytes.

Standards and Internal Standards. Our goal was to quantitate monophenolic metabolites of naphthalene, fluorene, phenanthrene, and pyrene (Figure 1). Most of these metabolites were commercially available, the exceptions being 4-hydroxyfluorene, 2-hydroxypyrene, and 3-hydroxypyrene, which fortunately have been reported to be minor metabolites.[14,48] Consequently, we used the commercially available standards in our method development.

Using mass spectrometry as the detection method makes it possible to use stable isotope-labeled internal standards in order to maximize precision and accuracy. Since acquiring stable isotope-labeled analogues of all analytes did not appear to be feasible for our studies, we decided to purchase or synthesize at least one labeled monophenol derived from each of the four parent hydrocarbons. Perdeutero-1- and 2-naphthol are commercially available. An analogue of 1-hydroxypyrene labeled with six carbon-13 atoms is available from the National Cancer Institute. 2-Hydroxyfluorene-d_9 and a mixture of hydroxyphenanthrene-d_9 isomers were synthesized in our laboratory. The synthetic route is shown in Figure 2 using 2-hydroxyfluorene-d_9 as an example. Completely deuterated parent hydrocarbons are commercially available. These were acylated with acetic anhydride and stannic chloride (Friedel−Crafts, method A) or formylated with α,α-dichloromethyl methyl ether (method B) to give aryl methyl ketones or aldehydes, which were then treated with *m*-chloroperoxybenzoic acid (Baeyer−Villiger oxidation) to give the corresponding acetoxy- or formyloxyarene. Base hydrolysis of the esters provided the deuterated phenols. This method yielded 2-hydroxyfluorene-d_9, since electrophilic substitution of fluorene occurs predominantly at this position.[49] Electrophilic substitution of phenanthrene is more complex, and generally a mixture of isomers is obtained, the composition depending upon the reaction conditions.[50] Mixtures that contained 1-, (and/or 9-), 2-, and 3-hydroxyphenanthrene-d_9 as the major components were obtained, as determined by the relative retention times of the PFB derivatives compared to nondeuterated standards (Figure S-2, Supporting Information). The amounts of d_0-isotopomers in the hydroxyfluorene-d_9 and hydroxyphenanthrene-d_9 preparations were less than 0.1%.

A potential problem with the use of deuterium-labeled phenols as internal standards is the possibility of deuterium loss by enol−keto tautomerization during extraction or derivatization. Although carbon-13-labeled internal standards would eliminate this problem, obtaining these for naphthols, hydroxyfluorenes, and hydroxyphenanthrenes was not feasible at the time our studies were initiated. Therefore, in order to investigate the possibility of deuterium exchange, we dissolved 2-naphthol-d_7 and a mixture of hydroxyphenanthrene-d_9 isomers in pH 7 buffer, let the solution stand for 3 days at room temperature, extracted the mixture, and then subjected the extract to the derivatization procedure. In another experiment, we incubated the deuterium-labeled metabolites with β-glucuronidase and arylsulfatase at pH 7 for 4 days at 37 °C. These procedures were similar to the extraction/derivatization of biological samples (see Experimental Section), with the exception that the incubation with buffer (3−4 days) was longer than the corresponding period (overnight) used for enzymatic deconjugation of the urine samples. This was done so that these conditions for potential deuterium exchange would be more extreme than the conditions used for the biological samples. The derivatized extracts were analyzed by LC/MS for isotopomers that could result from deuteron−proton exchange. Interestingly, deuterium loss occurred to a large extent only with 2-naphthol-d_7, and it was converted to the d_6-isotopomer, with further exchange occurring only at a very slow rate (Table S-2, Supporting Information). These results suggested a possible solution to the problem: it should be possible to convert 2-naphthol-d_7 to 2-naphthol-d_6 and use this as the internal standard. For preparative purposes, triethylamine proved to be an effective catalyst in refluxing ethanol/acetonitrile solvent (Figure S-1, Supporting

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Table 4. Urinary Excretion of PAH Metabolites in Smokers and Nonsmokers[a]

analyte	ng/mL mean (range) smokers ($N = 21$)	ng/mL mean (range) nonsmokers ($N = 22$)		pmol/mg creatinine[d] mean (range) smokers ($N = 21$)	pmol/mg creatinine[d] mean (range) nonsmokers ($N = 21$)	
2-naphthol[b]	14.3 (1.7−51.1)	2.44 (BLQ−17.5)	$p < 0.0001$	110 (41−190)	19.0 (3.5−88.5)	$p < 0.0001$
1-hydroxyfluorene	0.96 (BLQ−4.57)	all BLQ	$p = 0.0002$	5.41 (1.54−14.7)	0.97 (0.17−2.75)	$p < 0.0001$
2-hydroxyfluorene	1.60 (0.20−6.62)	0.11 (BLQ−0.33)	$p < 0.0001$	9.50 (3.22−24.4)	0.90 (0.36−2.18)	$p < 0.0001$
sum of 1- and 2-hydroxyfluorene				14.9 (5.29−39.1)	1.86 (0.53−3.75)	$p < 0.0001$
1-hydroxyphenanthrene	0.28 (0.029−1.34)	0.095 (BLQ−0.46)	$p = 0.0067$	1.54 (0.36−4.86)	0.69 (0.16−1.62)	$p = 0.0007$
2-hydroxyphenanthrene	0.16 (0.017−0.70)	0.04 (BLQ−0.15)	$p = 0.0019$	0.85 (0.35−1.91)	0.29 (0.14−0.76)	$p < 0.0001$
3- + 4-hydroxyphenanthrene[c]	0.450 (0.066−2.13)	0.063 (BLQ−0.23)	$p = 0.0025$	2.31 (0.86−6.48)	0.52 (0.24−1.92)	$p < 0.0001$
sum of hydroxyphenanthrenes				4.69 (1.78−11.5)	1.49 (0.62−4.17)	$p < 0.0001$
1-hydroxypyrene	0.33 (0.029−2.00)	0.061 (BLQ−0.23)	$p = 0.0044$	1.59 (0.32−4.04)	0.39 (0.093−0.77)	$p < 0.0001$

[a] Comparison of smokers and nonsmokers by unpaired *t*-test. For determining means and ranges of creatinine-normalized values, concentrations below the lower limit of quantitation (BLQ) were assigned the value LOQ divided by the square root of 2. [b] The isomeric metabolite 1-naphthol coeluted. Since the MS response was ~5% of that of 2-naphthol, and human urine concentrations of the two metabolites are generally similar, the analytical results should be fairly close to those of 2-naphthol for most samples. See text, Table 3, and Figure S-5. [c] The relative response of 4-hydroxyphenanthrene was ~25% that of 3-hydroxyphenanthrene. Concentrations of 3-hydroxyphenanthrene generally exceed those of 4-hydroxyphenanthrene in human urine by 5−10-fold[18,21] so this sum should be a good estimate of the concentration of 3-hydroxyphenanthrene for most samples. [d]Concentrations normalized to creatinine to adjust for differences in urinary flow.

Information). Loss of one deuterium atom was complete after a 2-h reflux period, with only a few percent loss of a second deuterium. The relative abundance of *m/z* 143, corresponding to the unlabeled isotopomer, was 0.04%. Syntheses of the internal standards and the experiments on deuterium exchange are described in the Supporting Information. Subsequent to the development of our method, ^{13}C-labeled 1-, 3-, and 4-hydroxyphenanthrenes and 2-naphthol have become available commercially.

Sample Preparation. The phenolic metabolites of PAHs are excreted in urine largely as the glucuronide conjugates, although small amounts of the sulfates and free phenols may also be excreted.[6,22] Consequently, the first step in nearly all reported methods for PAH metabolite determination involves incubation with β-glucuronidase and arylsulfatase to convert conjugates to the free phenols. Most published methods have used an enzyme mixture isolated from *Helix pomatia*.[15,17,23,26] We found that using β-glucuronidase from *E. coli* and sulfatase from *Aerobacter aerogenes* gave cleaner extracts (fewer emulsions) and better recovery of analytes than with samples incubated with the *Helix* enzymes. Following deconjugation, the free phenols are extracted with a 90:10 mixture of pentane/ethyl acetate. The extracts are then evaporated and converted to the PFB derivatives by a phase-transfer procedure using pentafluorobenzyl bromide and tetrabutylammonium bromide as the catalyst. The derivatives are extracted into pentane, the pentane extracts are evaporated, and reconstituted in methanol for LC/MS/MS analysis.

Analyte Stability and Extraction Recovery. Phenolic compounds are susceptible to oxidation, and some investigators have added antioxidants to prevent losses from occurring during extraction procedures or chromatography in PAH metabolite assays.[51] In addition, some investigators have added surfactants to samples in order to minimize losses.[52] Initially we utilized aqueous instead of urine calibration standards, since all urine samples contain PAH metabolites. Surprisingly, recoveries (determined from stable isotope-labeled internal standard peak areas) were greater from spiked urine than from spiked water! Furthermore, recoveries from an "artificial urine" (see Experimental Section) developed as a blank matrix for preparation of standards were greater than from water and were generally similar to recoveries from real urine specimens (Figure 6). On the assumption that the losses were due to adsorption on surfaces of extraction glassware, inhibited by surfactants in the urine samples, Triton X-100 was added to aqueous standards, but this had little effect. However, addition of antioxidants (α-tocopherol, BHA, quercetin, gallic acid) did improve recoveries. The hydroxyfluorenes and hydroxyphenanthrenes were most susceptible to oxidation. It was found that losses were occurring both during the evaporation and derivatization steps, and addition of a small amount of gallic acid to the extracts prior to evaporation and derivatization was effective for inhibiting losses. (Figure S-4, Supporting Information). Recoveries of labeled internal standards ranged from 72 to 83%. Final extracts of derivatives in methanol were stable for at least 24 h at room temperature in autosampler trays and for at least 5 days at −20 °C.

Chromatography. Our goal was to separate as many of the isomeric metabolites as possible with a reasonably short chromatographic run time. Phenols derived from different hydrocarbons could be distinguished by mass, but isomeric compounds

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could not, since MS/MS produced the same product ions. Each parent hydrocarbon may be ring-hydroxylated to isomeric monophenolic metabolites: two for naphthalene, four for fluorene, five for phenanthrene, and three for pyrene (Figure 1). All are reported to be human metabolites. We evaluated several columns, and of these, the best separation of the isomeric PFB derivatives was achieved with a Thermo-Hypersil PAH Green column (Figure 7). The three commercially available monophenolic hydroxyfluorene isomers (1-, 2-, and 3-hydroxyfluorene) were all separated. 4-Hydroxyfluorene has been detected in human urine, but of the four isomers, it appears to be the least abundant.[14] 9-Hydroxyfluorene appears to be a minor metabolite,[14] and being an aliphatic alcohol, it predictably did not derivatize under our conditions. The PFB derivatives of 1- and 2-naphthol were not separated. Sensitivity for 1-naphthol was much lower than that for 2-naphthol by a factor of ~20, as determined by integration of SRM chromatograms of derivatized pure standards injected separately, apparently due largely to inefficient fragmentation of 1-naphthol anion under the CID conditions. Since the isomeric metabolites are generally excreted in comparable amounts in human urine,[53] the concentrations determined by our method should be fairly close to those of 2-naphthol for most samples. In support of this, 2-naphthol concentrations in 18 urine samples determined by LC/MS/MS were similar to those determined by GC/MS/MS, for which the isomeric naphthols were well separated (see below, Figure 8, Table 3, and Figure S-5). Of course, if determination of both metabolites were important, this would be a limitation of our method. The PFB derivatives of 1- and 9-hydroxyphenanthrene had identical retention times, but since the 9-hydroxy isomer has been reported to be a minor metabolite in humans,[26,51] and the sensitivity for 9-hydroxyphenanthrene was only 30% of 1-hydroxyphenanthrene, the analytical results can be considered to be mainly 1-hydroxyphenanthrene concentrations. The PFB derivatives of 3- and 4-hydroxyphenanthrene were partially resolved and determined as a sum. Since concentrations of 3-hydroxyphenanthrene in human urine have been reported to generally exceed those of 4-hydroxyphenanthrene by 5−10-fold,[18,21] and since the relative response of 4-hydroxyphenanthrene was 25% of 3-hydroxyphenanthrene, analytical results should be a good estimate of 3-hydroxyphenanthrene concentrations for most samples. As with 2-naphthol, if separation of all isomeric metabolites were important, this would be a limitation of our method. 1-Hydroxypyrene was the only pyrene metabolite available to us; it has been reported to be the major metabolite.[7]

Performance of the Method. Since PAH metabolites are present in all human urine specimens due to environmental and dietary exposure, working standards were prepared in "artificial urine" (Experimental Section), by spiking with a stock solution prepared in methanol. To validate this approach, controls were prepared by spiking nonsmokers' urine with the analytes, and the calculated amounts were compared to the amounts expected based on the difference between spiked and nonspiked results. Calibration curves were prepared by linear regression with $1/X$ weighting. These were linear over the ranges 0.25−50 ng/mL for 2-naphthol and 0.01−5 ng/mL for the other analytes. Correlation coefficients for standard curves are in the Experimental Section. Artificial urine blanks, treated with β-glucuronidase and arylsulfatase and derivatized in the same manner as standards and urine samples were free from interfering substances (Figure 7A). Precision and accuracy, evaluated by analyzing nonsmokers' urine spiked with the analytes at concentrations spanning the expected ranges, were very good for all analytes except 3-hydroxyfluorene, which was erratic and for which reliable quantitation was not possible (Table 2). Fortunately, 2-hydroxyfluorene is the major fluorene metabolite,[14,15] and precision, accuracy, and sensitivity for it were excellent. Limits of quantitation (LOQ) were determined by analyzing spiked artificial urine, because the sensitivity of the method was good enough to determine concentrations below those found in urine of nonexposed persons, for most analytes (Table 2). We used the criteria of Shah et al.[54] to validate the method for precision and accuracy and to determine the LOQ. These are precision (CV) of ±15% and accuracy within ±15% of the expected amount, except at the lower limit of quantitation, for which ±20% is considered acceptable.

To further validate the method, derivatized extracts of urine samples were analyzed by both LC/MS/MS and GC/MS with NICI. Without putting much effort into optimizing chromatographic separations or mass spectrometric parameters, we were able to quantitate some metabolites using GC/MS. 1- and 2-naphthol PFB derivatives were easily separated, as were the derivatives of all three available hydroxyfluorene isomers. 2-Hydroxyphenanthrene was well separated from the other isomers; 1- and 3-hydroxyphenanthrene coeluted; sensitivity was poor for 4- and 9-hydroxyphenanthrene. We were unable to detect the 1-hydroxypyrene PFB derivative even at column temperatures considerably above those satisfactory for the other analytes. We evaluated both SIM and SRM, and as expected, SRM gave cleaner chromatograms of urine extracts. Using SIM, the hydroxyphenanthrene PFB derivative peaks were lost in baseline noise, but it was possible to quantitate 2-hydroxyphenanthrene using SRM (Figure 8). A fourth peak was present in the m/z 181−153 SRM chromatograms of urine extracts, but not in standards. We speculate that this is due to the presence of 4-hydroxyfluorene, for which a standard was not available. For those analytes that could be quantitated by both methods, there was generally a good correlation between LC/MS/MS and GC/MS/MS results. (Table 3, Figure S-5).

Determination of PAH Metabolites in Urine of Smokers and Nonsmokers. For the metabolites of all four PAHs, concentrations were significantly higher in smokers compared to nonsmokers (Table 4). Interestingly, the differences were most pronounced for hydroxyfluorenes, and for these two groups of subjects, there was no overlap in creatinine-normalized excretion of 2-hydroxyfluorene. In contrast, there was some overlap in concentrations between the two groups for the widely used PAH biomarker 1-hydroxypyrene, and for the other PAH metabolites, as has been previously reported.[55] The relatively high specificity of fluorene for tobacco smoke compared to other PAHs was suggested by data from the literature on emissions from various sources[8−11] and supports the value of measuring metabolites of several PAHs in studies of exposure, as suggested by other

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investigators.[6,16–20] There were also large differences in 2-naphthol concentrations between the two groups. It has been proposed that urinary naphthols may be particularly appropriate biomarkers for inhalation exposure to PAHs and that 2-naphthol is a better biomarker than 1-naphthol for inhalation exposure.[19]

In summary, a method for the simultaneous determination of ring-hydroxylated metabolites of four PAHs using liquid chromatography with electron capture atmospheric pressure chemical ionization mass spectrometry has been developed. Applicability of the method to studies of PAH exposure in smokers has been demonstrated, and sensitivity is adequate for determination of several PAH metabolites in urine of persons without unusually high exposures.

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