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Determination of selected monohydroxy metabolites of 2-, 3- and 4-ring polycyclic aromatic hydrocarbons in urine by solid-phase microextraction and isotope dilution gas chromatography–mass spectrometry

Christopher J. Smith*, Charisse J. Walcott, Wenlin Huang, Vince Maggio, James Grainger, Donald G. Patterson Jr.

Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Toxicology Branch, 4770 Buford Highway, MS F-17, Atlanta, GA 30341, USA

Abstract

Eighteen monohydroxy polycyclic aromatic hydrocarbon metabolites (OH-PAHs) representing polycyclic aromatic hydrocarbons (PAHs) containing up to four rings in human urine have been measured. The method includes the addition of carbon-13 labeled internal standards, enzymatic hydrolysis, and solid-phase microextraction followed by gas chromatography with high-resolution mass spectrometry. By using response factors calculated with the carbon-13 labeled standards, results are presented for calibration, relative standard deviations and analyte levels from an unspiked human urine pool. The method detection limits ranged from 0.78 ng/l for hydroxyphenanthrenes to 15.8 ng/l for 1-hydroxynaphthalene, and the recoveries ranged between 6% for hydroxychrysene and 47% for 1-hydroxypyrene. The relative standard deviation was lowest for 3-hydroxyphenanthrene at 2.4% and went up to 18.7% for 6-hydroxychrysene. The method was calibrated from 10 to 1200 ng/l. Eleven of the 18 metabolites were found in background pooled urine samples. This validated method is a convenient and reliable tool for determining urinary OH-PAHs as biomarkers of exposure to eight PAHs.
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1. Introduction

Because they are a chemical class found in essentially all environments, several polycyclic aromatic hydrocarbons (PAHs) have been extensively studied for their ambient levels, toxic effects and metabolism in model mammalian systems. The distribution and physical properties of PAHs vary widely from naphthalene to PAHs containing more than 10 fused benzene rings [1–5]. Their chemical properties vary, especially the toxicological results upon bioactivation [6–10]. The exposure to PAHs has generally been assessed from the metabolites or adducts found in blood and urine samples.

During the past 2 decades, many different methods of measuring urinary metabolites have been reported. High-performance liquid chromatography (HPLC) has been used with immunoaffinity columns and synchronous fluorescence spectroscopy by Weston et al. [11] to measure 1-hydroxypyrene (1-pyr) and

*Corresponding author. Tel.: +1-770-488-4166; fax: +1-770-488-4609.

E-mail address: cps6@cdc.gov (C.J. Smith).

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with fluorescence detection by Bentsen-Farmen et al. [12], who also looked at the tetrol metabolite of benzo[*a*]pyrene. HPLC fractions from solid-phase extraction (SPE) samples were collected and analyzed with solid-matrix luminescence by Ramasamy et al. [13]. Singh et al. reported the use of HPLC and fluorescence detection to determine hydroxypyrene plus its sulfate and glucuronide conjugates [14]. Earlier, Jongeneelen and co-workers used SPE and HPLC with fluorescence detection to measure the amount of urinary 1-pyr [15,16]. This method has been used by many groups to examine levels of 1-pyr in sample population studies [17–19]. Also, several groups have reported improved HPLC procedures for monohydroxy polycyclic aromatic hydrocarbon metabolite (OH-PAH) determinations [20–22].

Although use of HPLC methods has been widespread, interest has existed in the application of gas chromatography–mass spectrometry (GC–MS) to measure PAH metabolites. Some of the most extensive GC–MS work has been performed by Grimmer and co-workers [23,24]. Their methods include liquid–liquid extraction and derivatization before GC–MS analysis. Another method uses SPE with derivatization [25]. Overviews of investigations of PAH metabolites using HPLC and GC–MS can be found in summaries by Jongeneelen [26] and by Jacob and Grimmer [27].

In a recent communication Gmeiner et al. reported a screening method for urinary monohydoxy PAH metabolites [28]. Their procedure included the use of commercially available coated fibers to extract deconjugated OH-PAHs. With this solid-phase microextraction (SPME) technique, the OH-PAHs could be easily derivatized and analyzed by GC–MS. The sample preparation and extraction involves few steps, and the method can detect many OH-PAHs.

The objective of the research reported herein was to develop a validated analysis of urine for the 2-, 3- and 4-ring urinary OH-PAHs at exposure levels including background. Eighteen commercially available OH-PAHs derived from eight PAHs were included in the method. Following addition of carbon-13 labeled OH-PAH and enzymatic digestion, the sampling technique by Gmeiner et al. was used to extract the OH-PAHs. Separation and detection are performed with GC and high-resolution mass spectrometry (HRMS). Calibration curves, limits of detection and relative standard deviations (RSDs) are presented for the 18 OH-PAHs determined. Applying the method to a pooled urine collection of nonoccupationally exposed people, 11 of the OH-PAHs are easily quantified with only the larger 4-ring PAH metabolites reported as not detected.

2. Experimental

2.1. Chemicals

The list of analytes and labeled standards is presented in Table 1. Standards for the OH-PAHs were obtained from Aldrich (Milwaukee, WI, USA), Midwest Research Institute (Kansas City, MO, USA), AccuStandard (New Haven, CT, USA) and Promochem (Wesel, Germany). Internal standards were $^{13}C_6$ labeled with 99% purity and greater than 98% isotopic purity. The labeled standards were obtained as follows: 3-flranC13 and 1-pyrC13 from TerraChem (Merriam, KS, USA), 3-phenC13 and 6-chryC13 from Cambridge Isotope Labs. (Andover, MA, USA), and 1-napC13 was synthesized at the Centers for Disease Control and Prevention. Stock solutions of the individual OH-PAHs were made up in acetonitrile, and working solutions of the 18 OH-PAH mixtures were made by combining the aliquots of the stock solutions and further dilution in acetonitrile. A working solution of 100 pg/μl per labeled standard was prepared. The solutions were kept in silanized vials and stored at −70°C. Argon was used to displace the air in the vial headspace. Except as noted, all other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Urine donations were pooled from an anonymous volunteer group. To prepare samples for enzymatic hydrolysis, 3 ml of the urine pool and 10 μl of the 100 pg/μl labeled standard solution was added to 5 μl of β-glucuronidase/arylsulfatase (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 5 ml of 0.1 *M* sodium acetate buffer, pH 5.5. Magnetic stirring bars that had been extensively cleaned were placed in the sample vials, and the vials were capped

Table 1
List of native analytes, isotopically labeled standards, abbreviations, method detection limits (MDLs) and results from analysis of pooled urine (3 ml sample)

No.	Analyte	Abbreviation	MDL (ng/l)	Pooled urine (ng/l)
1	1-Hydroxynaphthalene	1-nap	15.8	44
2	2-Hydroxynaphthalene	2-nap	10.5	173
3	2-Hydroxyfluorene	2-fluor	1.5	734
4	3-Hydroxyfluorene	3-fluor	1.5	330
5	1-Hydroxyphenanthrene	1-phen	0.78	3
6	2-Hydroxyphenanthrene	2-phen	0.78	12
7	3-Hydroxyphenanthrene	3-phen	0.78	41
8	4-Hydroxyphenanthrene	4-phen	0.78	45
9	9-Hydroxyphenanthrene	9-phen	0.78	38
10	3-Hydroxyfluoranthene	3-flran	1.23	1.8
11	1-Hydroxypyrene	1-pyr	1.6	17
12	1-Hydroxybenzo[*c*]phenanthrene	1-bcp	1.38	ND
13	2-Hydroxybenzo[*c*]phenanthrene	2-bcp	1.38	ND
14	3-Hydroxybenzo[*c*]phenanthrene	3-bcp	1.38	ND
15	1-Hydroxybenz[*a*]anthracene	1-baa	1.32	ND
16	3-Hydroxybenz[*a*]anthracene	3-baa	1.47	ND
17	3-Hydroxychrysene	3-chry	1.68	ND
18	6-Hydroxychrysene	6-chry	1.68	ND
19	$^{13}C_6$-1-Hydroxynaphthalene	1-napC13		
20	$^{13}C_6$-3-Hydroxyphenanthrene	3-phenC13		
21	$^{13}C_6$-3-Hydroxyfluoranthene	3-flranC13		
22	$^{13}C_6$-1-Hydroxypyrene	1-pyrC13		
23	$^{13}C_6$-6-Hydroxychrysene	6-chryC13		

ND indicates level below MDL.

with a crimp seal and PTFE septa. The buffered samples were then incubated for 2 h at 37°C.

2.3. Sample extraction

All SPME apparatus were obtained from Supelco (Bellefonte, PA, USA). The extraction protocol by Gmeiner et al. was used with minor modification [28]. The fibers used in the SPME extraction were 85 μm coated polyacrylate. Each fiber was preconditioned at 300°C for 2 h, and before each use the fiber was desorbed in an offline GC injector inlet for 3 min at 290°C. A hydrolyzed urine sample was placed on a heater/stirring plate set at 40°C and stirred at the point of vortex formation. The SPME fiber was inserted through the septum into the liquid sample and allowed to remain for 45 min. To remove any liquid sample remaining on the fiber after extraction, the fiber was placed for 45 s into an oven set at 100°C. For the on-fiber derivatization, the fiber was then inserted into the headspace of a 1.5-ml silanized amber vial containing 15 μl of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and an argon atmosphere. The derivatization was carried out at 60°C for 1 h. The completeness of the derivatization step was checked by monitoring the underivatized OH-PAHs by using the molecular ion or the fragment upon loss of CHO. Derivatization produced better detection limits, and the derivatized analytes did not coelute. While awaiting analysis, the fibers could be placed in individually sealed plastic bags and stored in a refrigerator overnight.

When used in headspace applications, SPME fibers generally last for more than 100 extractions. Using direct immersion in a urine matrix, our investigations found that the fiber performance decreased at around the fifteenth run. Moreover, carryover was found after seven to nine runs. Therefore, each SPME fiber was used only five times to ensure acceptable results.

2.4. GC–MS conditions

The chemical analyses of the extracts were performed on a Finnigan MAT 95 high-resolution mass spectrometer (Bremen, Germany) with an Agilent Technologies 6890 gas chromatograph (Palo Alto, CA, USA). The GC injector and transfer line were kept at 290°C. The injector was operated in the splitless mode for 2 min, and a 1 mm I.D. glass liner was used. The chromatography column (J&W Scientific, Folsom, CA, USA) was a DB-5MS of 25 m×0.25 mm I.D., and 0.25 μm film. With a constant He flow of 1 ml/min through the column, the oven temperature was held at 100°C for 2 min, ramped to 160°C in 4 min, ramped from 160 to 295°C in 14 min, and then held at 295°C for 0.5 min.

The mass spectrometer was configured to monitor the molecular ions from the derivatized analytes using electron impact at 48 eV. This electron energy produced more intense signals from the molecular ions than the more commonly used 70 eV energy. The ion source temperature was 260°C. Similar ion intensities were generated from the molecular ion and from loss of a methyl group. Four time windows were set for the multiple ion detection at a resolution of 10 000 (10% valley). The chromatograms were analyzed by using the software provided by the mass spectrometer manufacturer.

2.5. Urine pools

Urine donations from nonoccupationally exposed individuals were collected and divided into pooled samples. One pool was used as a background level example, and one pool was spiked with all 18 OH-PAHs at 800 ng/l each. Thus, the spiked pool contained the spiked amount of OH-PAH plus background amounts of OH-PAH that were deconjugated enzymatically before extraction. The pools were transferred as 3-ml samples and stored at −70°C until needed.

3. Results and discussion

3.1. Chromatography

A difficult challenge in the analysis of OH-PAHs is the separation of a number of isomers. While naphthol may have two unique isomers (at the 1- and 2-positions), larger PAHs may be hydroxylated to produce many positional isomers, such as hydroxybenz[*a*]anthracene, which has 12 isomers. Confounding the problem from positional isomers, structural isomers among PAHs exist. To obtain sufficient resolution to minimize interferences and reasonable analysis times, we focused on the 2-, 3-, and 4-ring OH-PAHs that were commercially available. For each of the four multiple ion detection windows, Fig. 1 contains mass chromatograms from SPME extractions of urinary OH-PAHs. Separation of all 18 OH-PAHs was achieved even for the hydroxylated isomers of chrysene, benz[*a*]anthracene and benzo[*c*] phenanthrene at mass 316.13.

Using HRMS at 10 000 resolution, there were some minor interferences in the mass chromatograms in the low mass channels that were not attributable to metabolites of PAH. At higher masses there were resolved peaks indicating possible OH-PAH from outside our target list in Table 1.

Knowledge of human metabolic PAH products and their profiles and frequencies for specific PAHs is limited. In choosing to examine OH-PAHs, it is not possible to accurately predict which carbons on the PAH will be hydroxylated and their relative distributions. A few of the resolved peaks in the higher mass channels may be additional OH-PAHs; therefore, caution is warranted in the use of chromatograms to determine exposure assessment on the basis of a targeted list.

3.2. Isotope dilution calibration

Our initial experiments to determine calibration curves did not include isotopically labeled standards; we used several less common OH-PAHs as internal standards (surrogates), such as trihydroxyanthracene and 2-bcp. The extraction variability among the structural isomers was large, and differences in extraction of the positional isomers were not accurately reflected by the surrogates. For example, 1-bcp and 3-chry were spiked at the same levels, but the relative recovery among several replicate extractions varied up to 50%. Generally, the variability between isomers ranged between 10 and 20%. The use of isotopically labeled standards provided more precise correction of the variations in the SPME extraction. Acquiring labeled standards for all 18 OH-PAHs was

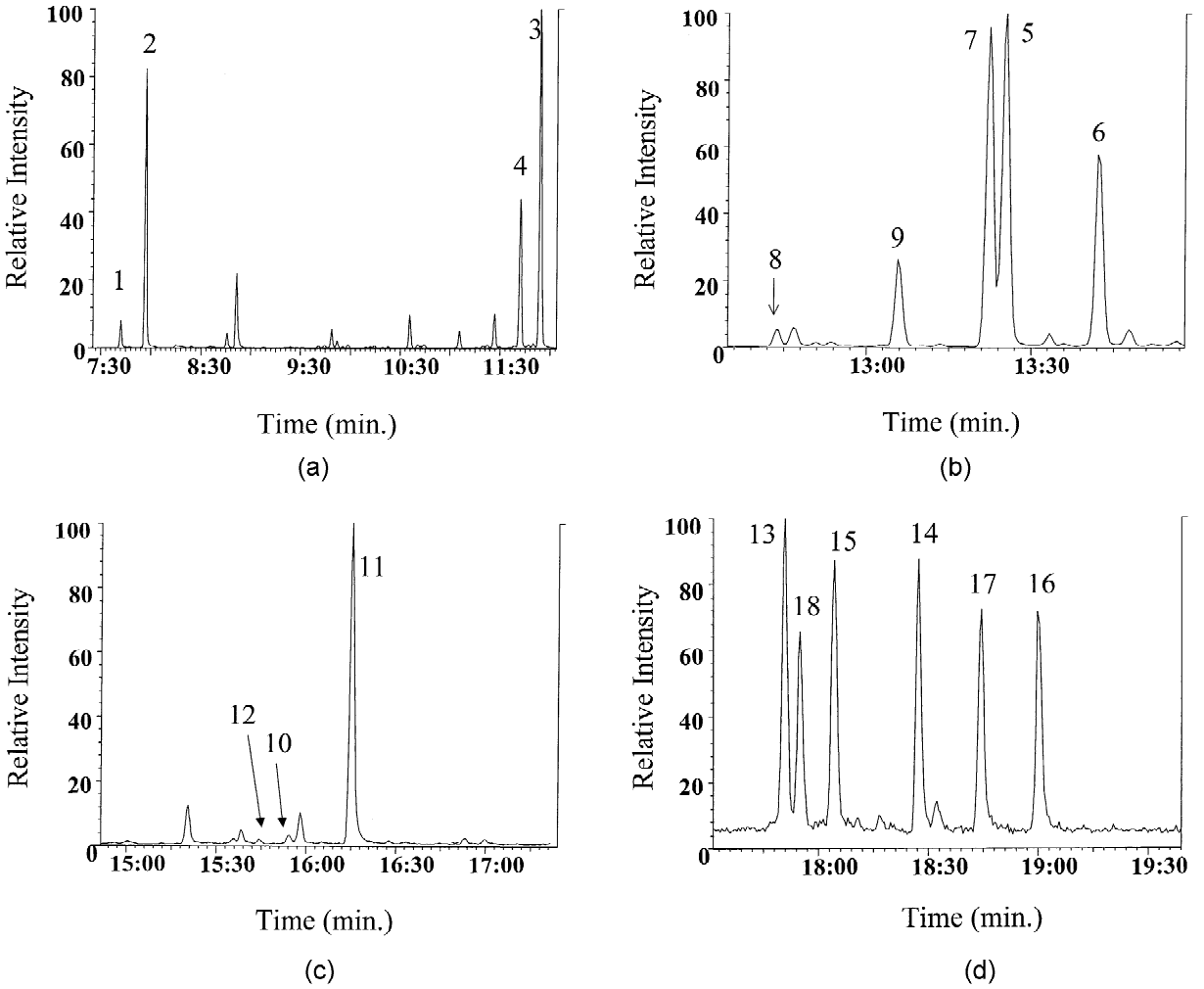

Fig. 1. Mass chromatograms from an SPE extraction of urine. (a)–(c) Chromatograms from a background pooled urine sample. (d) Background pooled urine sample spiked at 2 ng/l with the analytes which had non-detectable levels. Peak assignments correspond to the numbering in Table 1. The relative intensity was scaled to the largest peak in each time window.

cost-prohibitive; therefore, we chose a few labeled standards to cover each of the mass spectrometric time windows. 1-napC13 was used as the standard for the hydroxynaphthols and hydroxyfluorenes; 3-phenC13 referenced the hydroxyphenanthrenes; 3-flranC13 and 1-pyrC13 referenced their respective unlabeled compounds, and 6-chryC13 was used for the calibration of the hydroxychrysenes, hydroxybenz[*a*]anthracenes and hydroxybenzo[*c*]phenanthrenes.

Both spiked water samples (water, enzyme and buffer) and spiked urine samples (with acetate buffer and no enzyme) were used to generate calibration data. Samples of blank urine may contain small percentages of unconjugated OH-PAHs that may cause the low end of the calibration to be skewed [14]. Thus, the water samples were used in the final preparation of the calibration curves. The use of water samples for calibration of analytes in a urine matrix may often produce disparate results, but the labeled compounds absorbed onto the SPME fiber adequately correct for recovery changes.

For the calculation of recovery, external calibration by syringe injection of standards into the GC–MS system was used. Recoveries ranged from lows of 6% for the hydroxychrysenes to near 47% for

1-pyr and the hydroxyphenanthrenes. The SPME technique in combination with isotope dilution produces acceptable quantification. Using isotopic labels, determinations of unknown sample concentrations were performed by obtaining response factors and then calculating directly from the known response curves. This technique ensures better accuracy and precision when small variations in extraction time, temperature, and pH may disturb the equilibrium during the SPME protocol.

The calibration data were response factors (target analyte area/isotopic standard area) versus the concentration (ng/l) of the OH-PAH. The resulting curves were fit with software from the instrument manufacturer using linear regression with weighting factors given by the inverse concentration. By weighting the regression in this manner, the lower concentration data points were given more importance. All response curves exhibited a linear fit from 50 ng/l to 1200 ng/l, with R^2 values from 0.93 to 0.99. The response curves producing lower R^2 values were from 2-fluo, 3-fluo, 1- and 3-Baa, and 1- and 3-Bcp. These analytes lacked a C-13 labeled standard in their structural isomer group, and variations in the sample preparation were not as closely reflected by their reference compounds. Comparing positional isomers, the 3-phen response curve produced an R^2 of 0.99 while the other isomers such as 2-phen had R^2 values of 0.97 to 0.98. Thus, the methodology used was slightly sensitive to the positional isomers and quite sensitive to differences in the structural isomers.

Background contamination complicated the calibration of the naphthols. There was a small background level in the blank samples of purified water, and a sporadic increase due to contamination from the local environment. The sporadic increases were not directly traceable to a particular origin, but nearby building construction and renovation most likely contributed. All other analytes did not appear in the blank samples.

3.3. Limits of detection and quality control (QC) charts

Using the SPME protocol with on-fiber derivatization, excellent limits of detection were achieved. The method limit of detection (MDL) for each analyte is presented in Table 1. The MDLs were calculated as $3S_0$, where S_0 is the extrapolated value of the standard deviation as the concentration approaches zero [29]. The MDLs ranged from 0.78 to 15.8 ng/l for the hydroxyphenanthrenes and 1-nap, respectively. The background contamination of 1-nap and 2-nap caused their MDLs to be corrected to higher values. By calculating the MDL using variance of sample analysis, the skill and precision of the analyst throughout each step in the protocol can be reflected in the results. Therefore, to limit additional sources of variance, only one analyst performed the sample preparation for the calibration.

Means and variances for the urine pools were examined from QC charts. To characterize the QC samples, at least 15 runs were used to monitor the long-term precision and accuracy. Fig. 2 contains a QC chart for 1-pyr from 3 ml samples of the spiked urine pool ($n=15$, 800 ng/l). The analyte in Fig. 2 has an isotopically labeled standard, which provided excellent statistics with an RSD of 6.3%. The RSD values for analytes with labeled standards ranged from 2.4% (3-phen) to 18.7% (6-chry). Using the QC charts, trends in the QC samples and outliers can be identified as potential problems. For future analysis, an analytical run, defined as a set of unknowns, QCs, and standards, may be accepted or rejected based upon the QC criteria.

3.4. Pooled background samples

Although occupational and environmental exposures are often measured, a reference range for each OH-PAH is useful for comparing baseline exposure with the extent of exposure above baseline. The results from a pooled sample are presented in Table 1. Data from this pool are not generalizable to the US population and cannot be traced to a single individual. From the pooled sample, the levels of detectable OH-PAH ranged from 1.8 to 734 ng/l. A report of not detected (ND) indicated a level below the MDL. The pooled results are presented in ng/l of urine, but for individual results it has been found that adjustment of OH-PAH levels to creatinine excretion is worthwhile [30]. In the upcoming year, we will analyze a set of NHANES+ (National Health and Nutrition Examination Survey) samples to establish

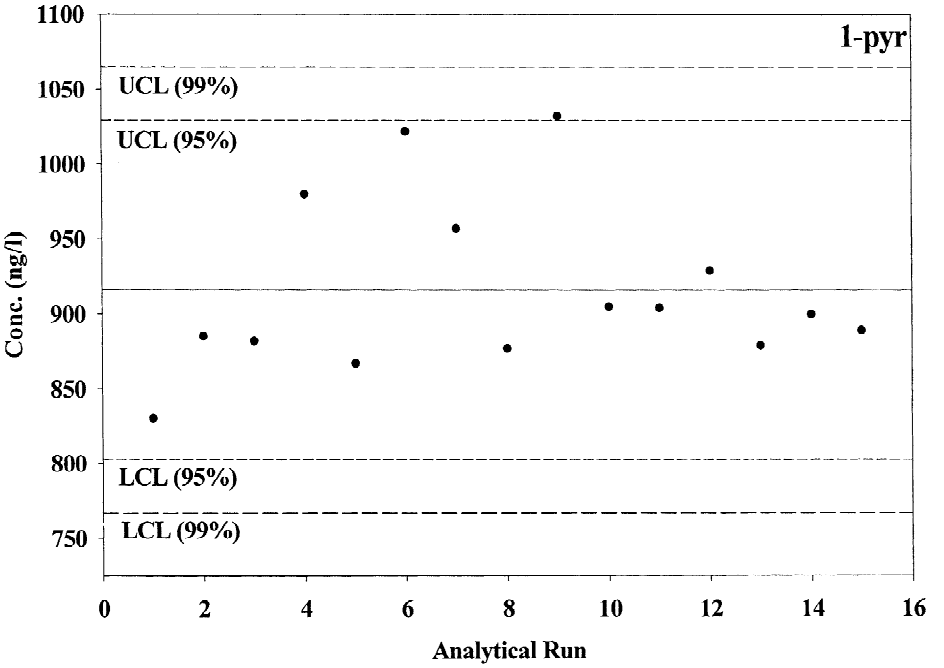

Fig. 2. Quality control chart for 1-pyr of a urine pool spiked at 800 ng/l. When QC values deviate outside the 99th confidence interval or showed a trend, the analytical run was rejected and corrective action taken. The mean value for 1-pyr was greater than 800 ng/l due to exogenous OH-PAHs in the pooled urine.

baseline values for the US population aged 6 years and older.

4. Summary

A method has been developed for the determination of metabolites of PAH in a human urine matrix. SPME sample preparation provides adequate results when used with isotope dilution mass spectrometry. Quality assurance measurements provided validation and aided in the detection of potential problems. The low MDL and large dynamic range make the method suitable for analyzing background OH-PAH exposure levels and studies of personal or occupational exposure.

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Christopher Smith obtained his Ph.D. in physical chemistry at the University of Colorado in Boulder, CO, USA, working at the Joint Institute for Laboratory Astrophysics with the National Institute of Standards and Technology. He then joined the research group of Dr. Edward Yeung at Iowa State University and Ames Laboratory, Ames, IA, USA, where he developed interest in bioanalytical separations and capillary electrophoresis. Since 1996 he has been with the Toxicology Branch of the Division of Laboratory Sciences at the Centers for Disease Control and Prevention in Atlanta, GA, USA. His current work includes analysis of PAH exposures in humans and applications of capillary electrophoresis in environmental toxicology.