Short Communication

Improved Method for Determination of 1-Hydroxypyrene in Human Urine

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Abstract

We have developed an improved method for the analysis of human urine for 1-hydroxypyrene (1-HOP), an accepted biomarker of polycyclic aromatic hydrocarbon uptake. This method takes advantage of commercially available 96-well format devices, which expedite sample preparation before quantitation by HPLC with fluorescence detection. In addition to improved speed of analysis, which is critical for the application of this assay in molecular epidemiology studies, the method described here uses an internal standard, 1-hydroxybenz[*a*]anthracene, improved sample preparation methods, and optimized HPLC and fluorescence detection conditions. The resulting method for analysis of 1-HOP is sensitive (detection limit, 0.05 pmol/mL urine), accurate (as determined by known addition of 1-HOP to urine), and precise [relative SD (RSD), 4.13%]. A longitudinal study of 1-HOP levels in the urine of 10 nonsmokers showed considerable day-to-day (mean RSD, 55.1 %) and week-to-week (mean RSD, 38.2 %) intra-individual variation, indicating the necessity for multiple sampling in studies concerned with relatively small differences in polycyclic aromatic hydrocarbon exposure. (Cancer Epidemiol Biomarkers Prev 2004;13(7):1261–4)

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a diverse group of environmental carcinogens formed during the incomplete combustion of organic matter (1). PAHs are believed to play an important role as causes of human cancer, particularly in certain occupational settings and in cigarette smokers (2-6). Various biomarkers, including PAH-DNA adducts, PAH-protein adducts, and urinary metabolites of PAHs, have been used to assess human uptake and/or metabolic activation of these carcinogens (7, 8). Among these, urinary 1-hydroxypyrene (1-HOP) is firmly established as a useful biomarker of PAH uptake, and is found to be clearly elevated in occupational settings with high PAH exposure (9, 10). 1-HOP is a urinary metabolite of the non-carcinogen pyrene, which always occurs in PAH mixtures that include carcinogens, such as benzo[*a*]pyrene (1).

We are involved in several studies that will generate a sizeable number of urine samples to be analyzed for 1-HOP, and concluded that it was necessary to modify existing analytic methods to increase speed of analysis. Since the first description of quantitation of 1-HOP in human urine by HPLC-fluorescence, numerous modifications of the methodology have been described (9-24). These include improvements in sensitivity, coupled column and immunoaffinity methods for analyte enrichment, various methods of standardization, and use of immunoassays and gas chromatography-mass spectrometry. None of these methods was fully satisfactory for our purposes. Therefore, we developed a modified HPLC-fluorescence method using a 96-well format for increased efficiency of sample handling.

Subjects and Methods

Collection of Urine. First morning urine (50 to 80 mL) was collected from 10 nonsmokers, 8 male, on 7 consecutive days, then, starting 1 week later, once weekly for 6 weeks. There were no lifestyle restrictions other than lack of tobacco smoking. The samples were collected in 4 oz. polyethylene specimen collection jars and kept at room temperature until being brought to the laboratory later that day. No preservative was added.

Analysis of Urine for 1-HOP. One milliliter of the urine sample was shaken and aliquoted into a 1.5-mL Eppendorf tube (Fisher Scientific, Pittsburgh, PA). Sodium acetate buffer (0.1 mL, 1.0 mol/L, pH 5.0) was added to adjust the urine pH to 5. Then 1-hydroxybenz[*a*]anthracene (1-HOBaA, 1 ng; National Cancer Institute Chemical Carcinogen Reference Standard Repository, Kansas City, MO) in 10 μL isopropyl alcohol, and 20 μL of a suspension of 2,000 units of β-glucuronidase and 16,000 units of sulfatase from *Helix pomatia* (Roche Diagnostics Corp., Indianapolis, IN), were added. The mixture was incubated overnight with

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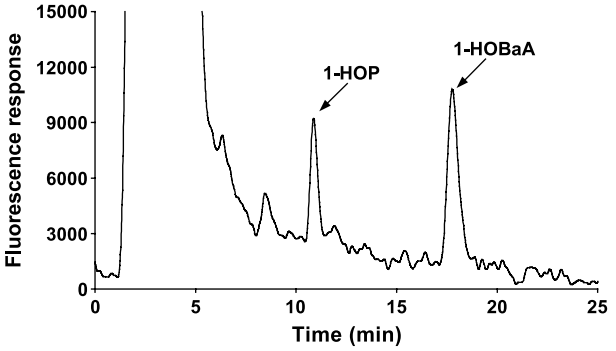

Figure 1. Chromatogram obtained on HPLC analysis with fluorescence detection (excitation 344 nm, emission 400 nm) of the 1-HOP fraction from a nonsmoker's urine.

shaking at 37°C. A Versaplate 96-well solid phase extraction system (Varian, Inc., Walnut Creek, CA) was used for sample preparation before HPLC analysis. It consisted of 96 individual polyethylene tubes, each containing 100 mg of 120 μm C_{18} packing (Varian 7550201C), a 96-well collection plate consisting of 96 1.3 mL round conical bottom wells (Varian SN400042), a 96-well Micromat Closure device (Varian SN400067), and a vacuum manifold. Sixteen of the removable cartridges were placed on a separate 24-port vacuum manifold (Alltech Associates, Inc., Deerfield, IL) equipped with individual stopcocks. The cartridges were primed with 1 mL of methanol and 2 mL of H_2O. The cartridges were maintained wet and the urine samples were applied (including, most importantly, any precipitate formed during enzyme treatment). The cartridges were washed with 1 mL of methanol-1% aqueous formic acid (1:1). This eluant was discarded. These cartridges were then placed in the 96-well device. The remaining cartridges, typically 60 to 70, were conditioned and the urine was applied in the same way. When all cartridges were in the 96-well device, two 0.4-mL portions of methanol were applied to each cartridge. The eluants were collected in the 96-well collection plate in which any unused spots were covered by sealing tape. The cartridges were eluted until they were dry. The solvent in the 96-well plate was concentrated to dryness at 40°C in a Speedvac Model SVC-200 (ThermoSavant, Holbrook, NY). The Speedvac was covered with aluminum foil to protect the samples from light. Fifty microliters of methanol were added to each well and the plate was sonicated briefly. Then, 50 μL of 50 mmol/L K_2HPO_4 buffer (pH 7) were added to each well. The 96-well plate was briefly sonicated and then, with its septum cover, was placed in an HPLC well plate autosampler (Model 1100 WPALS G1367A; Agilent Technologies, Wilmington, DE). Twenty-five microliters of each 100 μL sample were injected on a 150 × 4.6 mm 300PAH 5μ C_{18} column (Vydac, Hesperia, CA), equipped with a guard column (Vydac), and eluted isocratically at 1.2 mL/min with 60% methanol in H_2O. The temperature of the column was maintained at 35°C. The fluorescence signal was monitored using a Model RF10AXL detector (Shimadzu Scientific Instruments, Inc., Columbia, MD) using the analytic flow cell. Excitation was at 344 nm and emission at 400 nm. Solvents were degassed and continually sparged with He. 1-HOP eluted at 11 min and 1-HOBaA at 18 min. The HPLC column was cleaned with methanol after each 50 to 60 injections. Data were collected with a PeakSimple data system (Alltech Associates).

Results

Our modified method for analysis of 1-HOP in urine uses 1-HOBaA as internal standard. We did not detect this metabolite in more than 100 human urine samples analyzed without addition of internal standard nor has it been reported in the literature as a constituent of human urine. Experiments comparing the chromatographic properties and recoveries of 1-HOP and 1-HOBaA showed its suitability as an internal standard. A typical HPLC-fluorescence chromatogram of 1-HOP in a nonsmoker's urine is illustrated in Fig. 1.

Precision was determined by analyses of duplicate aliquots of a nonsmoker's urine sample, which was included with each set of urine samples analyzed over an 8-month period. The average values of the duplicate aliquots of the positive control sample in 30 sets of urine samples are illustrated in Fig. 2; mean 0.595 ± 0.0246 pmol/mL 1-HOP (N = 30). The relative SD (RSD) for assay precision was 4.13%.

Accuracy was assessed by the standard addition method. Duplicate 1-ml aliquots of a second positive control urine sample containing 0.487 ± 0.025 pmol/mL 1-HOP were spiked with 2.29, 4.59, 6.88, and 9.17 pmol 1-HOP. Analysis produced the results illustrated in Fig. 3. The added and measured levels of 1-HOP were highly correlated (R = 0.999) and the Y-intercept was 0.500 pmol/mL, in agreement with the amount of 1-HOP in the positive control sample.

The recovery of internal standard averaged 50%. The detection limit was 0.05 pmol 1-HOP/mL urine.

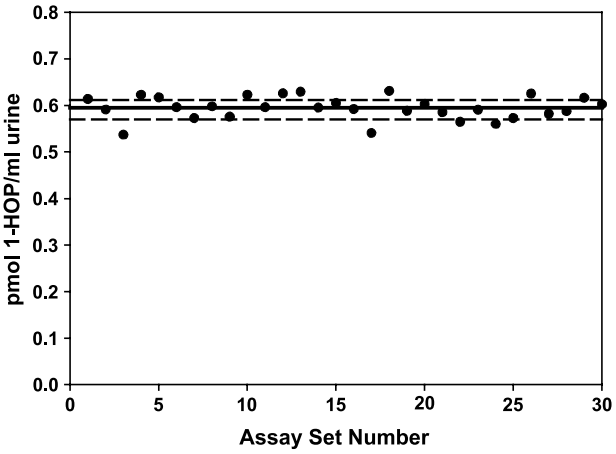

Figure 2. Levels of 1-HOP in a urine sample from one person, included as a positive control in the analysis of 30 sets of urine samples, done over an 8-month period. *Points*, average of analyses of duplicate samples included with that set. *Solid line*, mean (0.595 ± 0.0246 pmol/mL); *dashed lines*, SD. RSD = 4.13%.

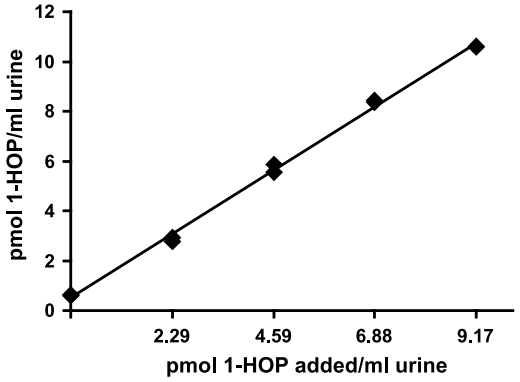

Figure 3. Results of a standard addition experiment in which various amounts of 1-HOP were added to urine and analyzed according to the scheme shown in Fig. 1. The *Y*-intercept corresponds to 0.500 pmol 1-HOP/mL urine, in agreement with the amount in the unspiked sample (see text for further details).

We assessed the longitudinal reproducibility of 1-HOP in the urine of 10 nonsmokers who collected urine samples daily for 7 days, then weekly for 6 weeks. The results are presented in Fig. 4A,B. The range of RSD values for day-to-day variation was 23% to 130% and the mean RSD ± SD was 55.1 ± 34.7% (Fig. 4A). The range of RSD values for week-to-week variation was 8.6% to 71% and the mean RSD ± SD was 38.2 ± 19.5% (Fig. 4B). Similar results were obtained when the 1-HOP data were expressed per milliliter urine.

Discussion

We have developed an improved method for analysis of 1-HOP in human urine. Major advantages of our method include speed of analysis of multiple samples, adequate sensitivity for analysis of relatively small urine samples, and the use of an internal standard, 1-HOBaA. The commercial availability and relative simplicity of handling of the 96-well device were crucial factors in our decision to use the approach described here. Our method is practical and should be widely applicable.

Several factors contributed to our relatively low detection limit, which allows the analysis of small urine samples. The inherent sensitivity of our fluorescence detector (signal to noise > 300 for Raman water), as well as our choice of emission and excitation wavelengths, gave maximum signal to noise for 1-HOP detection. The inclusion of material that precipitated from urine during enzyme hydrolysis was important because it contained considerable amounts of 1-HOP. The addition of formic acid to the eluting solvent in the solid phase extraction cleanup step produced cleaner and more reproducible HPLC traces. In addition, optimal HPLC conditions were guided by the critical studies of Sander et al. (25) on parameters affecting PAH separations.

Most methods reported in the literature do not use an internal standard (13). In our hands, 1-HOBaA, among several compounds that we investigated, was suitable based on its similar chromatographic properties to those of 1-HOP. Although we did not detect 1-HOBaA in human urine, it is possible that it could be present under conditions of high exposure to PAH. 1-HOBaA glucuronide, which was not available, would be an even better internal standard because it would account for any possible inefficiency in the β-glucuronidase hydrolysis step. However, we analyzed a positive control sample with each set of samples to verify enzyme activity for that set.

Diet is most likely the main contributor to 1-HOP in urine in nonsmokers without occupational exposure to PAH (26, 27). There were no dietary controls in our longitudinal study and this may account in part for the substantial day-to-day variation in 1-HOP excretion. The day-to-day (55.1%) and week-to-week (38.2%) intra-individual variations in 1-HOP excretion would not be important factors when comparing 1-HOP levels in highly exposed individuals (such as coke oven workers) to controls because 1-HOP levels would be elevated well beyond intra-individual variability (9). However, in lower exposure situations, such as cigarette smoking, longitudinal stability could be an important factor. Most studies show about a 2-fold increase in 1-HOP in smokers compared with nonsmokers, with varying

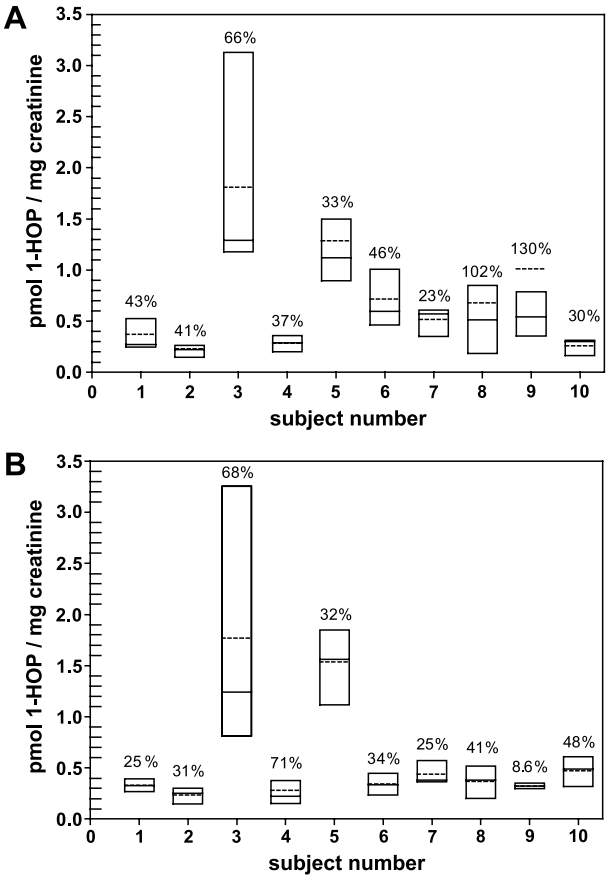

Figure 4. Intra-individual variation in levels of urinary 1-HOP in 10 nonsmokers who provided morning samples on each of 7 consecutive days (**A**) or on 1 day in each of 6 consecutive weeks (**B**). *Boxes*, 25th to 75th percentile values; *dotted lines*, mean; *solid lines*, median; *number above boxes*, RSD.

levels of statistical significance (7). In view of our results, it would be prudent to include multiple sampling in the design of studies relating cigarette smoking to 1-HOP levels.

In conclusion, we have developed an improved method for quantitation of 1-HOP in human urine. This method is practical, accurate, and precise and should find wide application in molecular epidemiologic studies of PAH uptake.

Acknowledgments

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