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Short communication

Improved high-performance liquid chromatographic method for the determination of polycyclic aromatic hydrocarbon metabolites in human urine

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Abstract

A reversed-phase HPLC method with fluorescence detection was evaluated for utility in determination of urinary metabolites of polycyclic aromatic hydrocarbons as biomarkers of environmental exposure. The method, which was developed for use in studies of high-level occupational exposure, was found to be unreliable for relatively low-level environmental exposures. The method was modified to include quantitation by standard addition in order to compensate for matrix effects at levels as low as 0.1 ng/ml. The standard addition modification increased both qualitative and quantitative performance, with recovery of 1-hydroxypyrene spikes improved from 164% to 114% at 0.36 ng/ml. The modified method was successfully applied in an environmental exposure study.

1. Introduction

The widespread presence of polycyclic aromatic hydrocarbons (PAHs) in the environment, along with the significance of their health effects, warrants a thorough investigation of the extent and dynamics of human exposure to this class of pollutants. As a measure of body burden, biomarkers provide a powerful and practical tool in the assessment of exposure. This is especially true for multimedia environmental contaminants such as PAHs. The biomarker measurement takes into account bioavailability and represents integrated exposure. The availability and use of such measurements can greatly reduce uncertainties in the assessment of exposure, thereby strengthening the assessment of risk.

The PAH metabolites 1-hydroxypyrene (1-OH-Pyr), 3-hydroxybenz[*a*]anthracene (3-OH-BaA), and 3-hydroxybenzo[*a*]pyrene (3-OH-BaP) have been used to classify occupational and high-level environmental exposures to PAH [1–6]. Selection of these particular PAHs was based on consideration of abundance, carcinogenicity, and range of vapor pressure. Determination of hydroxy-PAHs in urine samples is usually based on enzymatic hydrolysis of conjugates followed by reversed-phase HPLC with fluorescence detection and external standard quantitation [1,6,7], and this method has been shown to function well for 1-OH-Pyr levels indicative of occupational exposure. As HPLC determination of hydroxy-PAHs gains acceptance as a tool for

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characterizing exposure, the method is being applied to analysis of urine from individuals representing increasingly diverse exposure scenarios. Results from the current study suggest that modifications to the analytical approach may be warranted for applications to low-level environmental exposure. This paper describes a method of standard addition to the sample matrix that improves the method quantitation at very low levels.

2. Experimental

Standards of 3-OH-BaA and 3-OH-BaP were obtained from Chemsyn Science Laboratories (Lenexa, KS, USA). 1-Hydroxypyrene was obtained from Aldrich (Milwaukee, WI, USA). All standard solutions were made up in HPLC-grade methanol and stored in vials sealed with Teflon-lined caps at −20°C.

Urine samples were stored at −80°C until analysis, when each sample was thawed, and a 55-ml aliquot was transferred to an Erlenmeyer flask. Concentrated hydrochloric acid was used to bring the sample to pH 5, and 5 ml of 0.1 *M* pH 5 acetate buffer was added. At least 15 mg of β-glucuronidase (*Helix pomatia*, 338 000 units/g, Sigma, St. Louis, MO, USA) was added, the flask was capped, and the enzyme-activated sample was placed in a 37°C shaker bath overnight to hydrolyze the 1-OH-Pyr and 3-OH-BaP conjugates. After hydrolysis, the sample was pulled with vacuum through a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) prewetted with 5 ml of methanol and 10 ml of water. The cartridge was rinsed with 5 ml of water, and the sample was eluted with 10 ml of methanol into a graduated centrifuge tube. Sample volume was reduced under a stream of nitrogen and adjusted with methanol to 2.5 ml. The final solution was filtered through a 0.2-μm syringe filter and collected in a 4-ml amber vial with a PTFE-lined septum cap. For analysis by standard addition, two 0.5-ml aliquots of the sample extract were transfered to 1-ml autosampler vials, and one was spiked with 5 μl of 400 ng/ml standard solution to produce a spiked concentration of 4 ng/ml per component.

Samples were analyzed with an HPLC system consisting of a Waters 717 autosampler, 600MS solvent delivery system, 470 programmable fluorescence detector, and Millenium 2010 data system. The analytical column was a Nova-Pak (Waters) C_{18} column, 150 × 3.9 mm I.D. with 4-μm packing, thermostatted at 40°C. The solvent system was 50:50 (v/v) methanol–water (A) and methanol (B), held for 5 min at 100% A, then linearly programmed to 88% B at 40 min. The detector had a bandpass of 18 nm and was operated at a gain of 100, with initial emission and excitation wavelengths of 242 and 388 nm for 1-OH-Pyr, which were changed at 26 min to 290 and 402 nm to detect 3-OH-BaA, and at 30 min to 265 and 430 nm to detect 3-OH-BaP. The injection volume was 30 μl.

The initial analytes for this study were 1-OH-Pyr, 3-OH-BaA, and 3-OH-BaP. However, we discovered that the 3-OH-BaP standard was not stable, as evidenced by the appearance of two HPLC peaks over time, despite storage in the dark at −20°C. Because this finding called into question the integrity of samples not stored under nitrogen, and because qualitative examination of the samples resulted in no detection of 3-OH-BaP, all further discussion here is focused on 1-OH-Pyr and 3-OH-BaA.

3. Results and discussion

Previous applications of this method have generally focussed on 1-OH-Pyr levels greater than 1 ng/ml. Jongeneelen et al. [6] reported a 88 ± 9% recovery of 1-OH-Pyr at 8.7 ng/ml, and Buckley and Lioy [1] reported a 100 ± 24% recovery at 3.23 ng/ml. In 1993, the U.S. Environmental Protection Agency participated in a study of environmental exposure in which estimation of nonoccupational exposure to PAHs was desired. Preliminary work indicated that the levels of PAH metabolites in the samples would likely be less than 1 ng/ml, so we made an effort to determine and enhance the usefulness of the HPLC/fluorescence method at those levels.

Initial testing of instrument performance was carried out with standard solutions. Standards ranging from 0 to 20 ng/ml (corresponding to sample concentrations of 0 to 0.9 ng/ml) were used to generate calibration curves, which were found to be linear, with y-intercepts not significantly different from zero at the 95% confidence level. The limit of quantitation was determined from the standard deviation of seven injections of a 0.08 ng/ml standard to be 0.04 ng/ml, corresponding to a sample concentration of 0.002 ng/ml. The relative standard deviations for six injections of a 10 ng/ml standard (corresponding to a sample concentration of 0.45 ng/ml) were 6% for 1-OH-Pyr and 2% for 3-OH-BaA. The recoveries of standards spiked in water at 0.36 ng/ml and carried through the entire procedure were $90 \pm 9\%$ for 1-OH-Pyr and $69 \pm 7\%$ for 3-OH-BaA ($n = 3$).

Experiments with urine samples were somewhat more difficult because of the background material left after the sample cleanup. A representative chromatogram of an unspiked urine extract is shown in Fig. 1. Recoveries from urine samples spiked at 0.36 ng/ml were $164 \pm 26\%$ for 1-OH-Pyr ($n = 8$) and $81 \pm 12\%$ for 3-OH-BaA ($n = 5$) when calculated from external standard calibration. The high recoveries of 1-OH-Pyr were viewed as cause for concern. Urine samples from three different subjects were tested, with no differences detected. Peak integrations and assignments were rechecked manually.

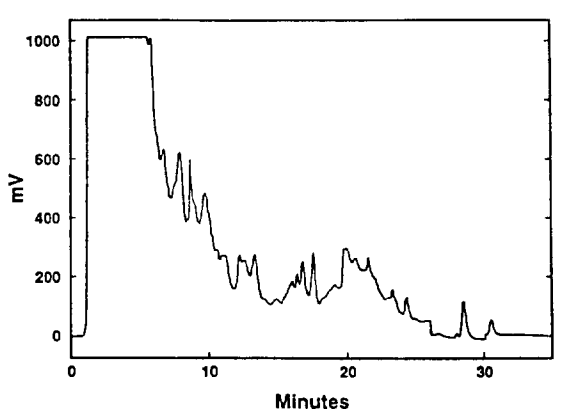

Fig. 1. Extract of unspiked urine sample after C_{18} SPE cleanup.

and we could find no explanation for the high recoveries of 1-OH-Pyr other than an unknown matrix effect altering the fluorescence response of the analyte.

We attempted to correct for this matrix effect by using standard addition quantitation. In a standard addition method, matrix effects are compensated for by determining the sample concentration based on response factors calculated from calibration standards added to the sample itself [8]. Because of the sample load and the amount of manual processing required, we were limited to single level standard additions. Representative chromatograms of a sample with and without standard spikes are shown in Fig. 2, along with a chromatogram of a standard corresponding to a sample concentration of 0.36 ng/ml. The recoveries of 0.36 ng/ml spikes calculated by the standard addition method were $114 \pm 23\%$ for 1-OH-Pyr ($n = 3$) and $86 \pm 12\%$ for 3-OH-BaA ($n = 4$). The number of replicates

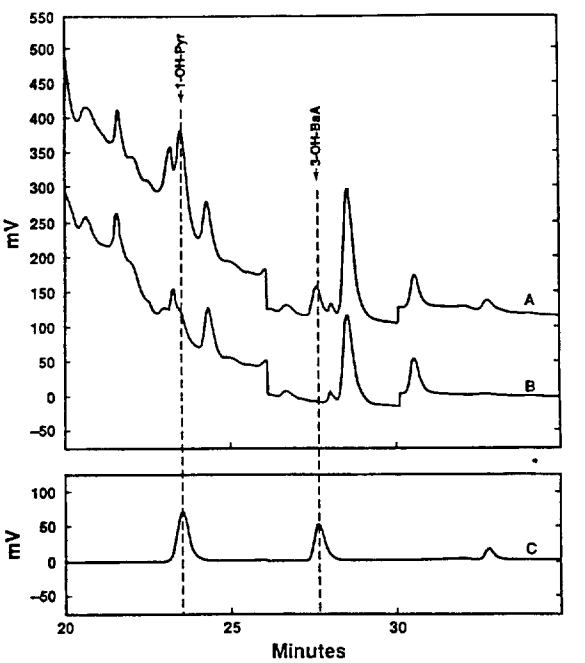

Fig. 2. Chromatograms of extract of urine sample not found to contain quantifiable amounts of 1-OH-Pyr and 3-OH-BaA, with (A) and without (B) 4 ng/ml standard addition, and (C) chromatogram of 8 ng/ml standard (corresponding to a sample concentration of 0.36 ng/ml).

Table 1
Recoveries of spikes at 0.36 ng/ml

Sample	Recovery (mean ± S.D.) (%)	
	1-OH-Pyr	3-OH-BaA
Spike in water	90 ± 9 (n = 3)	69 ± 7 (n = 3)
Spike in urine, external standard	164 ± 26 (n = 8)	81 ± 12 (n = 5)
Spike in urine, standard addition	114 ± 23 (n = 3)	86 ± 12 (n = 4)

was limited by a shortage of blank urine. A comparison of all of the spike recovery data is given in Table 1. In addition to improving quantitative results, the use of standard addition methodology improved the reliability of peak identifications. Analyte peaks can be picked out of a crowd of background peaks by comparison of chromatograms of spiked and unspiked extracts (Fig. 3).

The improved method was applied to two sets of samples collected in a U.S. Environmental Protection Agency field study of nonoccupational exposure. The results are shown in Table 2. Because of sample volume limitations, only four samples were analyzed in duplicate. The average percent difference between duplicates was 15% for 1-OH-Pyr. Only two of the duplicated samples were found to contain 3-OH-BaA, and the differences between those duplicates averaged 7%.

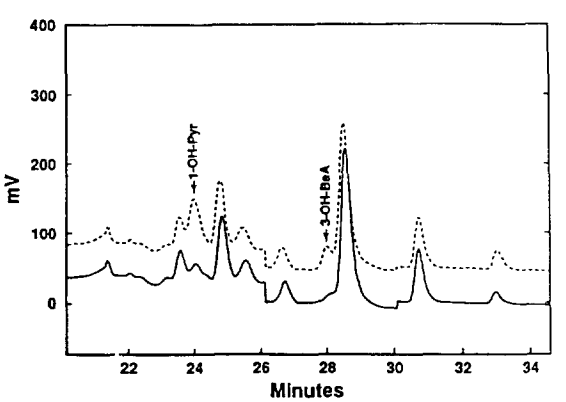

Fig. 3. Chromatograms of urine sample extract 1008A (bottom) found to contain 0.1 ng/ml 1-OH-Pyr, and extract with standard addition (top).

Table 2
Hydroxy-PAH concentrations in field-collected urine samples.

Sample	Concentration (ng/ml)	
	1-OH-Pyr	3-OH-BaA
1001	ND	ND
1002	0.65	ND
1003	0.1	ND
1004	0.1	0.1
1005	0.33	ND
1006	0.1	ND
1007	0.20	ND
1008	0.1	ND
1009	0.1	0.16
2003	0.89	0.1
2005	0.32	0.15
2006	0.26	0.17
2007	0.49	0.16
2009	1.7[a]	0.29

[a] Exceeded calibration range.
[b] ND = not detected at 0.1 ng/ml.

The sample-to-sample variability of the background interferences presents a difficulty for estimating the method detection limit. The value calculated from clean standards is clearly irrelevant, but a value calculated from replicate determinations of a single spiked standard may also not be helpful because of the variation in the background peaks. Analytes were identified in some relatively clean samples at levels estimated to be as low as 0.01 ng/ml, but quantitation was frequently difficult, so a conservative estimate of 0.1 ng/ml for the quantitation limit was made based on examination of the sample chromatograms.

4. Conclusions

The conventional reversed-phase HPLC method with fluorescence detection for the determination of PAH metabolites in urine was modified to overcome the difficulties in measurement of extremely low levels indicative of nonoccupa-

tional exposure. Through the application of standard addition quantitation, coupled with careful examination of the data for verification of automated peak integration, matrix effects were offset and data quality was improved for measurements at levels less than 1 ng/ml. The improved method was shown to be quantitative and potentially sensitive enough for use in classification of environmental PAH exposures.

Disclaimer

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